

DIFFERENTIAL ROLE OF PI-3KINASE p85 (α & β) REGULATORY SUBUNITS
IN MAST CELL DEVELOPMENT

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In loving memory of my dear father

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ABSTRACT

Subha Krishnan

DIFFERENTIAL ROLE OF PI-3KINASE p85 (α & β) REGULATORY SUBUNITS IN MAST CELL DEVELOPMENT

Stem cell factor (SCF) mediated c-Kit signaling, and downstream activation of Phosphatidylinositol-3 Kinase (PI-3K) is critical for multiple biological effects mediated by mast cells. Mast cells express multiple regulatory subunits of PI-3Kinase, including p85 α , p85 β , p50 α and p55 α . In the present study, we have examined the relationship between p85 α and p85 β subunit in mast cell development and show that loss of p85 α in mast cell progenitors impairs their growth, maturation and survival whereas loss of p85 β enhances this process. To further delineate the mechanism (s) by which p85 α provides specificity to mast cell biology, we compared the amino acid sequences between p85 α and p85 β subunits. The two isoforms share significant structural homology in the two SH2 domains, but show significant differences in the N-terminal SH3 domain as well as the BCR homology domain. To determine whether the c-Kit induced reduction in growth of mast cells is contributed via the N-terminal SH3 or the BCR homology domain, we cloned and expressed the shorter splice variant p50 α , and various truncated mutant versions of p85 α in p85 α deficient mast cells. We demonstrate both *invitro* and *invivo* that while the SH3 and the BH domains of p85 are dispensable for mast cell maturation; they are essential for normal growth and survival. In contrary to existing dogma on redundant functional role of PI-3K regulatory subunits, this study proves that p85 α and p85 β regulatory subunits of PI-3K have unique roles in mast cell development. We prove that p85 α deficiency impairs the expression of multiple growth, survival and maturation related genes whereas p85 β deficiency inhibits c-Kit receptor internalization and degradation. This novel finding on negative role of p85 β in mast cell development has

significant clinical implication, as this knowledge could be used to develop treatments for mast-cell-associated leukemia and mastocytosis.

Reuben Kapur, Ph.D., Chair

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INTRODUCTION

Mast cells originate from multipotent stem cells in the bone marrow (BM). These cells, which are critical mediators of inflammation, innate immunity and host defense are found most abundantly in areas that interface with the external environment, such as the skin, respiratory tract, lung tissue, gastrointestinal tract and the urinary system (Kirshenbaum, Kessler et al. 1991). Upon activation, mast cells release various mediators including histamine, leukotrienes, prostaglandins, serine proteases, and various cytokines, chemokines, and growth factors (Metcalf, Baram et al. 1997; Kinet 1999; Galli, Kalesnikoff et al. 2005). Mast cell products such as proteases and interleukin-10 play essential roles in inflammatory responses, as well as in tumor pathophysiology (Kalesnikoff and Galli 2008). Roles for mast cells have also been described in multiple sclerosis (Secor, Secor et al. 2000), rheumatoid arthritis (Lee, Friend et al. 2002) and coronary artery disease (Lee, Friend et al. 2002). However, recent studies challenge the dogma of a pathological role for mast cell activation, demonstrating its prominent role in early phases of innate immunity to pathogenic bacteria (Feger, Varadaradjalou et al. 2002).

I. Origin of mast cells

In 1878, Paul Ehrlich first observed mast cells in connective tissue; he concluded that these cells differentiated from fibroblasts (Ehrlich 1878). For the next century, mast cells were believed to be a connective tissue component that was derived from undifferentiated mesenchymal cells. Later, Kitamura established the hematopoietic origin of mast cells in mice. The origin of mast cells from multipotent bone marrow cells was demonstrated by transplant studies done in *W/W^v* mice, which are devoid of mast cells. These mice developed mast cells when they received bone marrow cells from normal

littermates (Kitamura, Shimada et al. 1977; Kitamura, Go et al. 1978; Kitamura, Yokoyama et al. 1981).

The origin of human mast cells from hematopoietic stem cells has been demonstrated *in vitro* and *in vivo* (Kirshenbaum, Kessler et al. 1991; Fodinger, Fritsch et al. 1994). Jamur et al. purified and characterized undifferentiated mast cell progenitors (MCps) from bone marrow of adult Balb/c mice. These progenitor cells were isolated by using two monoclonal antibodies, mAb-AA4 and mAb-BGD6, to obtain a homogeneous population of undifferentiated mast cells (AA4/BGD6⁺) from adult murine bone marrow (Jamur, Grodzki et al. 2005). These cells which were characterized as CD34(+), CD13(+), c-kit(+) and FcεRI⁻ exclusively gave rise to mast cells *in vitro* in the presence of IL-3 and SCF; and reconstituted spleen mast cells in lethally irradiated mice (Jamur, Grodzki et al. 2005). MCps were also identified in the bone marrow of adult C57BL/6 mice and were characterized by another group of researchers around the same time. These cells were characterized as Lin⁻c-Kit⁺Sca1⁻Ly6c⁻FcεRIα⁻CD27⁻β7⁺T1/ST2⁺ and gave rise to mast cells *in vitro*. These progenitor cells reconstituted mast cells when transplanted to mast-cell deficient mice (Chen, Grimbaldston et al. 2005). Collectively, these studies establish the origin of mast cells from multipotent hematopoietic cells.

II. Proposed mast cell developmental pathways

The developmental pathway of mast cell progenitors from hematopoietic stem cells has been a topic of controversy, and currently there are three proposed models for mast cell hematopoiesis.

It has been proposed that common myeloid progenitor (CMP) in bone marrow gives rise to all myeloid cells including mast cells (Janeway, 2001). Arinobu's model illustrated in Figure 1 is based on his identification of a population of basophil mast cell precursors (BMCPs) in the spleen of mice (Arinobu, Iwasaki et al. 2005). BMCPs ($\text{Lin}^- \text{Kit}^+ \text{Fc}\gamma\text{RII/III}^{\text{hi}} \beta 7^{\text{hi}}$) are derived from granulocyte/macrophage progenitors (GMP) in the bone marrow and are thought to be bipotent progenitors for basophil and mast cell lineages. Chen's model of MCps being derived directly from multipotent progenitor cells (MPPs) (Chen, Grimbaldston et al. 2005) agrees with an earlier report by Kempuraj et al. that suggested that mast cell progenitors develop from multipotent hematopoietic cells through a pathway distinct from other myeloid lineages (Kempuraj, Saito et al. 1999). MCps are derived from bone marrow, and then disseminated hematogenously to peripheral tissues.

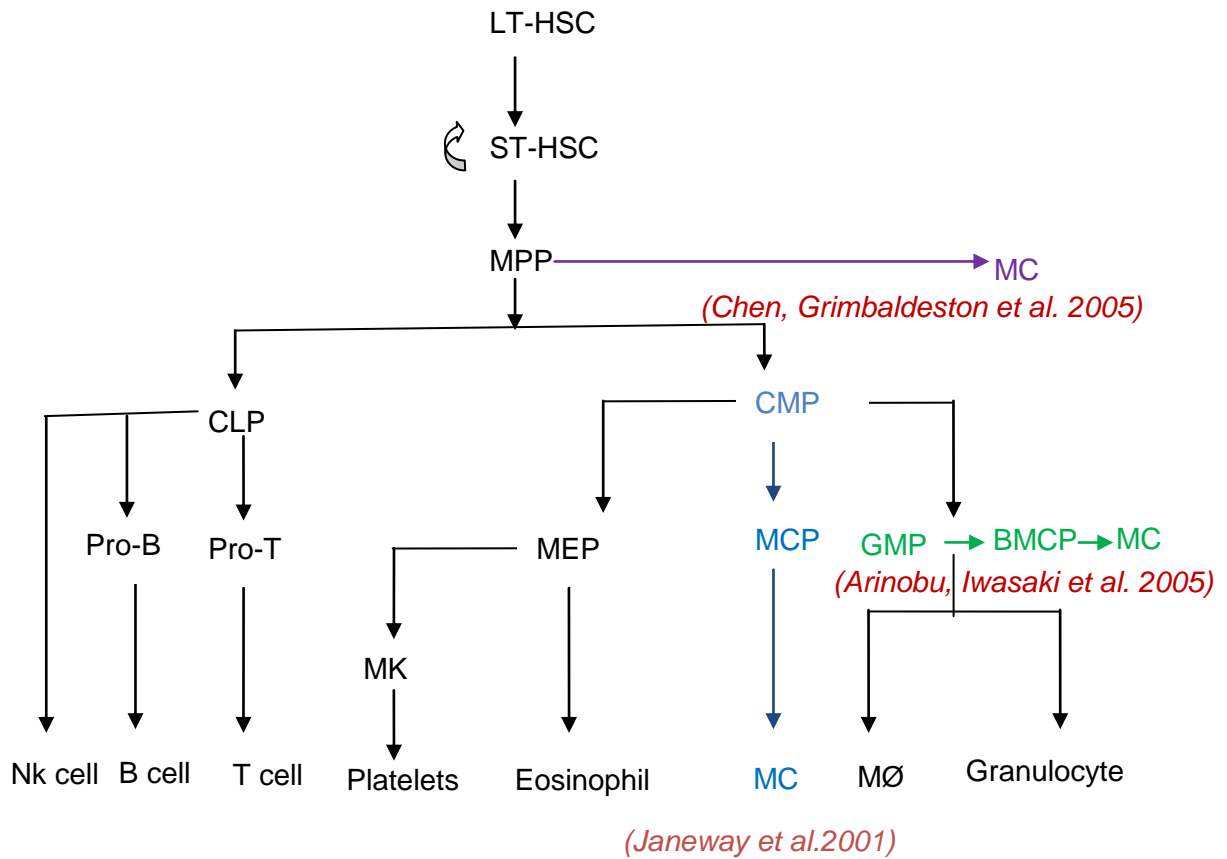


Figure 1. Mast cell developmental pathway. Three models have been proposed to explain the origin and maturation of mast cells from hematopoietic stem cells. Janeway (2001) proposed that mast cells are derived directly from a common myeloid progenitor (CMP) (represented in blue). Arinobu et al. (2005) proposed that mast cells are derived from basophil mast cell precursors (BMCP) in the spleen, which are derived from granulocyte/macrophage progenitors in the bone marrow (represented in green). A third model by Chen et al. (2005) proposed that mast cells are derived directly from multipotent progenitors in the bone marrow, rather from CMP or BMCP (represented in purple).

Abbreviations: LT-HSCs, long-term hematopoietic stem cell; ST-HSC, short-term hematopoietic stem cell; MPP, multipotential progenitor; CLP, common lymphoid

progenitor; CMP, common myeloid progenitor; MEP, megakaryocyte/erythrocyte progenitor; MCP, mast cell progenitor; GMP, granulocyte/monocyte progenitor; BMCP, basophil mast cell precursor; MC, Mast cell; MK, megakaryocyte; MØ, macrophages.

III. Mast cell trafficking to peripheral tissues

Although mature mast cells are never detected in circulation, the presence of MCps in blood and various tissues has been demonstrated by *in vitro* cultures and limiting dilution assays (Kasugai, Tei et al. 1995; Khalil, Luz et al. 1996; Gurish, Tao et al. 2001). A committed precursor for the mast cell lineage was first identified in fetal murine blood by Rodewald et al. (Rodewald, Dessing et al. 1996). These cells were characterized as Thy-1^{lo}c-kit^{hi}FcεRI⁻ and expressed mast cell specific proteases: mast cell carboxypeptidase A, mMCP-2 and mMCP-4. These cells give rise to mast cell colonies *in vivo* in the presence of IL-3 and SCF, and reconstitute a peritoneal mast cell population in mast-cell-deficient *W/W'* mice.

Additional support for the existence of circulating MCps was provided by Kitamura and Fujita, who used methylcellulose colony-forming assays to demonstrate that mast cell colonies can be formed from circulating mononuclear cells in the presence of growth factors including SCF and IL-3. Committed mast cells progenitors (CD34+/ FcεRI⁻) identified in human peripheral blood differentiated into mast cells in the presence of SCF *in vitro* (Kitamura and Fujita 1989; Kirshenbaum, Kessler et al. 1991; Rottem, Okada et al. 1994). These results suggested that, unlike other progeny of multipotent stem cells (erythrocytes, neutrophils, eosinophils and basophils) that leave the bone marrow after they differentiate, mast cells leave the bone marrow as immature but committed MCps (Galli 1990; Kitamura, Kasugai et al. 1993).

IV. Mast cell homing

Subsequently, Kitamura observed morphologically identifiable mast cells in the skin, stomach, peritoneal cavity and liver of mouse embryos (Kitamura, Shimada et al. 1979; Sonoda, Hayashi et al. 1983; Jippo, Morii et al. 2003). Several research teams have independently detected T-cell dependent, committed MCps in murine spleens, lymph nodes and mucosal surfaces (Crapper and Schrader 1983; Guy-Grand, Dy et al. 1984). Using methylcellulose colony-forming assays, Kasugai et al. showed the migration of mast cell progenitors from the blood to the small intestine during a *Nippostrongylus brasiliensis* infection (Kasugai, Tei et al. 1995). Pennock and Grecis tracked the generation and migration of MCps from bone marrow to blood to small intestine after infecting C57BL6 and NIH mice with *Trichinella spiralis* (Pennock and Grecis 2004). Thus, MCps, which originate from stem cells in the bone marrow migrate to the peripheral blood in immature form and complete their differentiation and maturation after invading mucosal and connective tissues (Kitamura 1989; Galli, Zsebo et al. 1994; Metcalfe, Baram et al. 1997; Galli, Maurer et al. 1999; Galli 2000; Galli, Kalesnikoff et al. 2005).

Although mast cells are present in all organs, trafficking of MCps from peripheral blood to various organs are driven by specific pathways. The homing and recruitment of mast cells to various tissues is regulated by complex network of protein interactions.

A. Scf

Binding of the ligand SCF to its c-Kit receptor provides critical signals for homing and recruitment of mast cells to various tissues. Mice that lack either c-Kit or SCF are almost completely devoid of mature mast cells in all tissues (Kitamura and Go 1979; Oku, Itayama et al. 1984; Galli and Kitamura 1987).

B. Integrins

Integrin heterodimer $\alpha 4\beta 7$ and its corresponding ligands, vascular cell adhesion molecule-1 (VCAM-1) and mucosal addressin cell adhesion molecule (MAdCAM1) are critical for maintaining a pool of MCps in the small intestine (Gurish, 2006). Mice deficient in integrin $\alpha 4\beta 7$, or those in which $\alpha 4\beta 7$ integrin or their ligands are blocked by antibodies, are devoid of MCps or matured mast cells in the intestine. VCAM-1 (which interacts with both $\alpha 4\beta 1$ and $\alpha 4\beta 7$ integrins), but not MAdCAM1 is essential for the recruitment of MCps into the lungs during pulmonary inflammation (Abonia, Hallgren et al. 2006). Integrin $\alpha IIb\beta 3$ mediates mast cell homing and retention in the peritoneal cavity; mice that are deficient in the glycoprotein IIb subunit of the $\alpha IIb\beta 3$ integrin have significantly reduced peritoneal mast cell populations (Shattil and Newman 2004; Berlanga, Emambokus et al. 2005).

C. Chemokines

In addition to integrins, chemokine receptors expressed by mast cell progenitors play a critical role in directing the migration of MCps from circulation into the tissues (Humbles, Lu et al. 2002; Abonia, Austen et al. 2005). Human mast cells express various chemokine receptors CXCR2, CXCR4, CCR3 and CCR5 (Ochi, Hirani et al. 1999). The interaction of chemokine receptor 2 (CXCR2) with its ligand is critical for directing MCps to the intestine (Abonia, Austen et al. 2005). A profound decrease in the mast cell progenitor population in the small intestine was reported in mice deficient in CXCR2 or those in which anti-CXCR2 was administered (Abonia, Austen et al. 2005). The role of CCR3 in mast cell homing has been identified in CCR3-deficient mice (Humbles, Lu et al. 2002). A significant (2- to 4-fold) increase in intra-epithelial mast cells is found in the tracheas of CCR3-deficient mice after an allergen challenge, which suggests that CCR3 is involved in the egress of mast cells from the mucosal intraepithelial compartment of

the lung (Humbles, Lu et al. 2002). These studies highlight the role of multiple factors—the integrins, the growth factors, and the cytokine receptors—in influencing tissue localization of MCps.

V. Mast cell subsets

After MCps are homed into various tissues, they develop into heterogeneous populations under the influence of different microenvironmental factors (Enerback and Lowhagen 1979; Bienenstock, Befus et al. 1985; Kitamura 1989; Galli, Zsebo et al. 1994; Metcalfe, Baram et al. 1997; Huang, Sali et al. 1998). Based on histologic, functional, compositional and pharmacologic regulatory properties, two types of mast cells, connective tissue mast cells (CTMC) and mucosal mast cells (MMC) have been defined in rodents (Enerback 1966; Enerback 1966; Bienenstock, Befus et al. 1983; Barrett and Metcalfe 1984; Katz, Stevens et al. 1985). CTMCs are predominantly found in the skin and peritoneal cavity, whereas MMCs are found mainly in the mucosal layer of the gut and lungs.

An important distinction between the CTMC and MMC subsets is the absolute dependence of MMCs on T-cell–derived factors for their development. Thymus-deprived (*nu/nu*) mice are completely deficient of MMC (Ruitenberg and Elgersma 1976).

From a histochemical perspective, MMC and CTMC differ in that CTMC contain proteoglycan heparin, large amount of histamine and carboxypeptidases; whereas MMC contain condritin sulfate, less histamine and carboxypeptidase.

The two mast cell phenotypes also exhibit functional differences. Upon IgE-induced activation, MMC mainly produce leukotriene C4; CTMC mainly produce prostaglandin

D2 (Kitamura 1989). Similar to rodents, humans have two distinct types of mast cells: MC_T and MC_{TC}. MC_Ts are predominantly found in the lungs and gastrointestinal mucosa; MC_{TC}s are most abundant in the skin and the gastrointestinal submucosa (Schwartz, 1987, Irani, 1986). Similar to MMC, the development of MC_T critically depends on T-cell-derived factors as seen by a significant reduction in their numbers in patients with immunodeficiencies (Irani, Craig et al. 1987). The two subsets can be distinguished on the basis of their protease content and secretory granules. MC_{TC} contains tryptase, chymase, cathepsin G, and carboxypeptidase, whereas MC_T express only tryptase (Irani, Schechter et al. 1986). In addition to exhibiting heterogeneity with respect to neutral protease content of the secretory granules, human mast cells also vary with respect to their cytokine content. MCT produce both IL-5 and IL-6, but MCTC produce only IL-4 (Bradding, Okayama et al. 1995). Despite such histochemical and functional differences, these cells reportedly are derived from a common lineage. It is also thought that the above phenotypes are plastic and interchangeable (Sonoda, Sonoda et al. 1986; Arinobu, Iwasaki et al. 2005).

VI. c-Kit

c-Kit, a member of the type III receptor tyrosine kinase subfamily, is a transmembrane protein expressed in mast cells, hematopoietic progenitor cells, melanocytes, germ cells and gastrointestinal pacemaker cells (Galli, Tsai et al. 1993). c-Kit is downregulated as all hematopoietic lineages mature except mast cells. Mast cells retain high levels of c-Kit expression throughout their maturity.

Normal mast cell development requires direct interaction between the c-Kit receptor (expressed in MCps and mast cells) and the ligand SCF secreted by fibroblasts and other cells in the microenvironment where mast cells develop. In mice, c-Kit is expressed

from two alternately spliced mRNAs, which, following protein glycosylation, give rise to products with molecular weights around 145kDa (Yarden, Kuang et al. 1987). c-Kit is encoded by the white-spotting (*W*) locus on chromosome 5, and the c-Kit ligand SCF is encoded by the steel (*S*) locus on chromosome 10 of mice (Chabot, Stephenson et al. 1988; Copeland, Gilbert et al. 1990). The mutations at the *W* locus abolish the c-Kit tyrosine kinase receptor on the cell surface or produce receptors with markedly deficient tyrosine kinase activity (Geissler, Ryan et al. 1988; Nocka, Tan et al. 1990). Mutations at the *S* locus result in the absence of the c-Kit receptor ligand, stem cell factor (SCF), or the production of abnormal forms of SCF (Flanagan and Leder 1990; Flanagan, Chan et al. 1991). Mice with mutations at the *W* locus (*W/W^v*) or the *S* locus (*S/S^f*) are profoundly deficient in mast cells (Kitamura, Go et al. 1978; Kitamura and Go 1979).

While several cytokines influence the growth, survival and maturation of mast cells, SCF and its interaction with the c-Kit receptor are critical for normal mast cell development and function.

A. Structure

c-Kit is characterized by the presence a signal sequence at its N-terminus, which is followed by five immunoglobulin (Ig)-like motifs, a transmembrane domain, and a cytosolic tyrosine kinase domain that is split into proximal and distal regions by an insert sequence. The second and third Ig-like motifs constitute a pocket for SCF binding (Zhang, Zhang et al. 2000). When SCF binds to the c-Kit receptor, the latter undergoes dimerization to initiate intrinsic tyrosine kinase activity. The fourth Ig motif of c-Kit contains the dimerization site, the deletion of which completely abolishes receptor dimerization and subsequent downstream signal transduction events.

SCF affinity for the c-Kit receptor depends on receptor dimerization, as indicated by accelerated ligand dissociation in the monomeric form of c-Kit and in c-Kit with defective dimerization sites (Blechman, Lev et al. 1995). SCF binding induces a conformational change to dimerize the receptor, which stabilizes the ligand-receptor interaction (Blechman, Lev et al. 1995). The juxtamembrane domain of c-Kit inhibits receptor dimerization and enzyme activity, maintaining an inactive conformation (Roskoski 2005). After initiation of kinase activity, various tyrosine residues in the cytoplasmic tail of the c-Kit receptor become phosphorylated, and function as docking sites for the Src homology2 (SH2) domain containing signal transduction molecules (Pawson 1995). The kinase domain of c-Kit is responsible for catalyzing and transferring a phosphate group from ATP to the substrate and activating them thus initiating downstream signaling (Roskoski 2005).

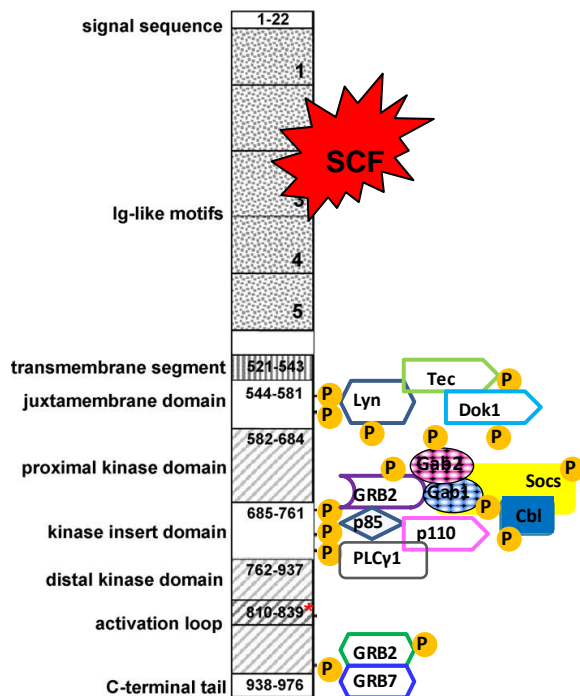


Figure 2. Schematic structure of c-Kit receptor and overview of the signaling

pathways activated upon SCF ligation to c-Kit. The distinct molecular domains

comprise three functional structures: NH₂-terminal extracellular, transmembrane and

COOH-terminal intracellular domains. The extracellular domain contains five

immunoglobulin-like repeats and the intracellular domain encodes two tandem repeats of

the enzyme catalytic domains. The juxtamembrane region binds to Src family members,

and changes in several residues within this region have been shown to result in

constitutive activation of c-Kit. The proximal kinase domain of c-Kit contains amino acid

residues involved in binding ATP. The kinase insert domain contains a tyrosine residue

that contributes to the binding of PI-3K. Asterisks indicate the position of aspartic acid-

814 in the second catalytic domain, and substitution of this residue to valine (D814V)

results in constitutive activation of c-Kit.

B. Function

Signaling cascades initiated by c-Kit stimulate mast cell functions related to differentiation, proliferation, survival and activation (Taylor and Metcalfe 2000). c-Kit and its ligand, stem cell factor (SCF) induce proliferation of mouse mast cells *in vitro* (Huang, Nocka et al. 1990; Martin, Suggs et al. 1990; Matsui, Zsebo et al. 1990; Nocka, Buck et al. 1990; Williams, Eisenman et al. 1990; Zsebo, Williams et al. 1990; Zsebo, Wypych et al. 1990) and *in vivo* (Tsai, Shih et al. 1991; Tsai, Takeishi et al. 1991). SCF-induced c-Kit kinase activity is essential for mast cell homeostasis, growth and differentiation of CD34⁺ human mast cell progenitor cells *in vitro* (Kirshenbaum, Goff et al. 1999). SCF is a potent chemotactic agent for MCps and mast cells, and also acts as a major factor responsible for adhesion of mast cells to connective tissue matrices (Dastyh and Metcalfe 1994; Nilsson, Butterfield et al. 1994; Dastyh, Taub et al. 1998). Thus, SCF plays a crucial role in the migration and homing of MCps to mucosal and connective tissues where they reside and terminally differentiate (Meininger, Yano et al. 1992; Okayama and Kawakami 2006). Survival of mature mast cells is also dependent on SCF (Yee, Hsiau et al. 1994; Metcalfe, Mekori et al. 1995; Da Silva, Reber et al. 2006). In addition to their role in triggering differentiation, maturation and migration of mast cells, SCF is also recognized as potent modifier of mast cell activation and secretion of mediators including tumor necrosis factors, proteolytic enzymes, glycosaminoglycans, and lipid mediators.

C. Negative regulation of c-Kit signaling

To maintain homeostasis, c-Kit signaling is attenuated after a period of time in mast cells. Miyazawa et al. reported polyubiquitination and degradation of c-Kit in response to SCF, which regulates c-Kit signaling in M07e cells (Miyazawa, Toyama et al. 1994). Unrestricted activation of c-Kit results in abnormal growth and survival of mast cells,

causing acute myeloid leukemia, systemic mastocytosis and gastrointestinal stromal tumors.

SCF stimulation, which initiates several signals for positive regulation of cell growth and proliferation, also initiates the phosphorylation and activation of c-Cbl ubiquitin ligase. c-Cbl is thought to associate with Src kinase in initiating the c-Kit degradation process, thus attenuating various intracellular signals (Levkowitz, Waterman et al. 1998; Lee, Wang et al. 1999; Miyake, Mullane-Robinson et al. 1999; Taher, Tjin et al. 2002; Masson, Heiss et al. 2006). Activated c-Cbl mediates the degradation of c-Kit through the proteasomal pathway (Zeng, Xu et al. 2005). Expression of a Cbl mutation (CblR420Q) in mice inhibited SCF-induced ubiquitination and internalization of c-Kit and led to mastocytosis and myeloid leukemia (Bandi, Brandts et al. 2009).

D. Abnormal c-Kit signaling

Although c-Kit is a critical molecule for mast cell development, certain activating mutations of this receptor, result in ligand-independent autophosphorylation that leads to constitutive activation of c-Kit, causing dysregulated cell growth and induction of tumorigenesis (Kitayama, Kanakura et al. 1995; Tsujimura, Morimoto et al. 1996).

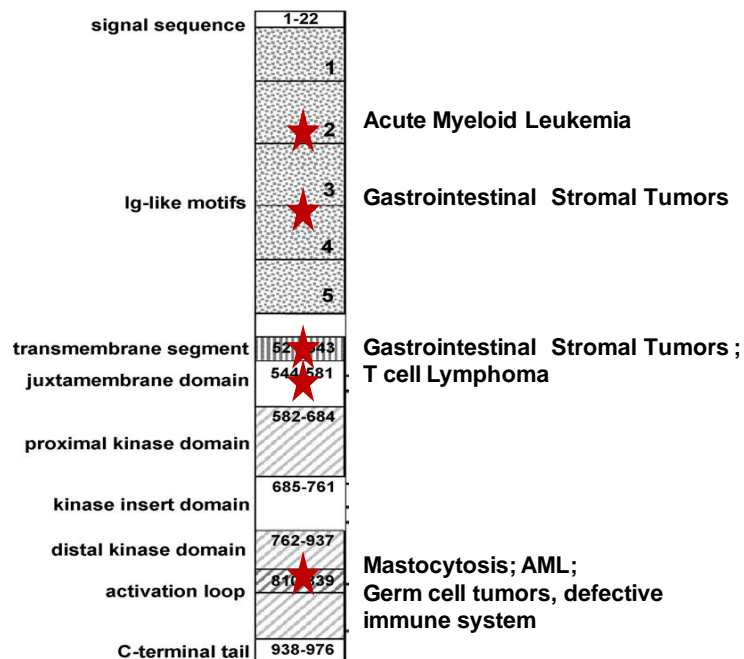


Figure 3. c-Kit structure and mutations found in human disorders. c-Kit mutations results in ligand independent consitutive activation of c-Kit resulting in human disorders. Mutations in c-Kit extracellular region (found in about 9%), and mutations in juxtamembrane domain (found in most 70%) of GIST patients and are effectively treated by imatinib. Mutations in c-Kit distal kinase domains (D816V) are typical of systemic mastocytosis, and are resistant to imatinib.

Activating mutations of c-Kit are classified as either “regulatory” or “enzymatic” (Longley, Reguera et al. 2001). The juxtamembrane domain (JMD) of c-Kit, which serves an autoinhibitory function, is mutated in several gain-of-function mutants of c-Kit. JMD mutations including deletions, point mutations, tandem duplications and combination deletion-point mutations have been associated with gastrointestinal stromal cell tumors (GISTs) (Duensing, Heinrich et al. 2004; Duensing, Medeiros et al. 2004). These mutations, which are attributed to genetic alterations in the JMD region that regulate the

kinase activity of c-Kit, are the “regulatory” type, and they might result in conformational changes in c-Kit that cause it to dimerize and activate.

Another set of c-Kit-activating mutations are referred to as the “enzymatic pocket type” mutation. These directly affect the sequence of c-Kit’s active enzyme site. This has been studied in humans (D816V), mice (D814V) and rats (D817V). Constitutively activating mutations of the c-Kit gene involve alterations of its phosphotransferase domain, which was reported to confer factor-independent growth in human mast cell leukemia (HMC-1), murine mastocytoma (P-815) and rat mast cell leukemia (RBL-2H3) (Furitsu, Tsujimura et al. 1993; Tsujimura, Furitsu et al. 1994). D816V mutation of the human c-Kit (homologous to D841V of mouse) has been associated with mast cell proliferative disorder, mastocytosis, acute myeloid leukemia and germ cell tumors (Tian, Frierson et al. 1999; Beghini, Peterlongo et al. 2000; Valent, Horny et al. 2001).

The aspartic acid (asp) residue encoded by the 816 codon of human c-Kit is located in the tyrosine kinase domain and is involved in ATP binding and subsequent phosphotransferase activity (Mol, Lim et al. 2003; Vendome, Letard et al. 2005). Amino acid substitution of Asp-816 to valine in human c-Kit stabilizes the kinase in its active conformation, thus resulting in ligand-independent activation of c-Kit (Mol, Lim et al. 2003; Vendome, Letard et al. 2005).

Interestingly, activation of c-Kit caused by this JMD mutation is due to constitutive dimerization of c-Kit in absence of SCF, whereas a D814V mutation induces SCF-independent growth without receptor dimerization (Kitayama, Kanakura et al. 1995; Tsujimura, Morimoto et al. 1996). Unlike gastrointestinal stromal tumors, which are treated effectively with Gleevac, systemic mastocytosis associated with a c-Kit D816V

mutation does not respond to Gleevac. This is thought to be due to the inability of the drug to bind to the ATP binding site, whose conformation is altered by a c-Kit point mutation at position 816 (Scheinfeld 2006). Currently there are no drugs on the market can specifically target the kinase domain mutants of c-Kit.

Previous studies report that PI-3K, which binds to the 719 tyrosine residue of murine c-Kit (721 a.a. in human c-Kit) through its regulatory subunit, substantially contributes to factor-independent abnormal growth in c-Kit–mutated cells (Chian, Young et al. 2001). PI-3K is constitutively activated in c-Kit (D814V)–induced myeloproliferative disorders, and treatment of these cells with the PI-3K inhibitor wortmannin specifically inhibits ligand-independent growth (Chian, Young et al. 2001). In hematopoietic neoplasms and tumors, PI-3K is reported to be persistently active, which might contribute to the abnormal growth of those cells (Vivanco and Sawyers 2002). Hadhimoto et al. reported that PI3-K plays an important role in ligand-independent growth and tumorigenicity in c-Kit (D814V) mutant cells (Hashimoto, Matsumura et al. 2003). The abnormal growth induced by this c-Kit (D814V) mutation can be suppressed by genetic disruption of the p85 α regulatory subunit of PI-3K (Munugalavadla, Sims et al. 2007). Alterations of c-Kit signaling was not observed when the p85 β regulatory subunit was disrupted in these cells, which suggests a unique role for the PI-3K regulatory subunits in the control of constitutively active c-Kit signaling.

In this thesis, we evaluated specific role of various PI-3K regulatory subunits in mediating c-Kit signals. Since there are no effective drugs available for the treatment of c-Kit (D814V)– related diseases, this information is likely to be important for the design of peptides that specifically target and correct the abnormal phenotype caused by this c-Kit (D814V) mutation.

VII. PI-3K (Phosphatidylinositol-3-kinase) and c-Kit signaling

PI-3K comprises a family of lipid kinases that are essential for the growth, differentiation, proliferation, survival and migration of mast cells. Structural characteristics and substrate specificity divide PI-3K into four classes: Class IA, IB, II and III (Fruman, Meyers et al. 1998; Wymann and Pirola 1998; Walker, Perisic et al. 1999). Class IA PI-3Kinase is heterodimeric kinases consisting of a regulatory subunit and a catalytic subunit (Sasaki, Suzuki et al. 2002; Okkenhaug and Vanhaesebroeck 2003). Class IA has three types of catalytic subunits: p110 α , p110 β and p110 δ . Of those three, p110 α and p110 β are expressed in many tissues, whereas p110 δ is expressed mainly in leukocytes. Five different proteins (p85 α , p55 α , p50 α , p85 β and p55 γ) have been identified to date as the Class IA regulatory subunits. The p85 α , p55 α and p50 α proteins are derived from mRNA splice variants encoded in the gene *Pik3r1*, while p85 β is encoded by *Pik3r2* and p55 γ is derived from *Pik3r3* (Fruman, Cantley et al. 1996). The regulatory subunits have several motifs implicated in protein-protein interactions. All the class IA regulatory subunits have two Src homology 2 (SH2) domains that bind phosphorylated tyrosine residues of various receptors and adaptor molecules (Sasaki, Suzuki et al. 2002; Okkenhaug and Vanhaesebroeck 2003). The inter-SH2 domain constitutively interacts with a specific domain of p110 catalytic subunit (Sasaki, Suzuki et al. 2002; Okkenhaug and Vanhaesebroeck 2003). The dual SH2 domains are functionally important because they recruit the p110 catalytic subunit to tyrosine-phosphorylated proteins at the cytoplasmic membrane (Sasaki, Suzuki et al. 2002; Okkenhaug and Vanhaesebroeck 2003). Furthermore, the interaction of the dual SH2 domains with the phosphotyrosine residue of c-Kit releases the p110-kinase activity that is normally blocked by complex formation between the regulatory and catalytic subunits (Sasaki, Suzuki et al. 2002; Okkenhaug and Vanhaesebroeck 2003).

In contrast to the dual SH2 domains, the N-terminal SH3 domain that precedes the Bcr homology (BH) domain is found only in the p85 α and p85 β isoforms by not in the 50 or 55 kDa subunits of PI-3K. Of its various regulatory subunits, *PI3kr1* protein products p85 α , p55 α and p50 α have been reported to be predominant in insulin-sensitive tissues, representing 80% of the total regulatory subunits. The p85 β regulatory subunit is present at lower levels (~30%) in these tissues (Ueki, Fruman et al. 2002; Ueki, Yballe et al. 2002). Although p85 α and p85 β are encoded by two different genes, they share 100% domain homology and 62% overall amino acid identity (Otsu, Hiles et al. 1991).

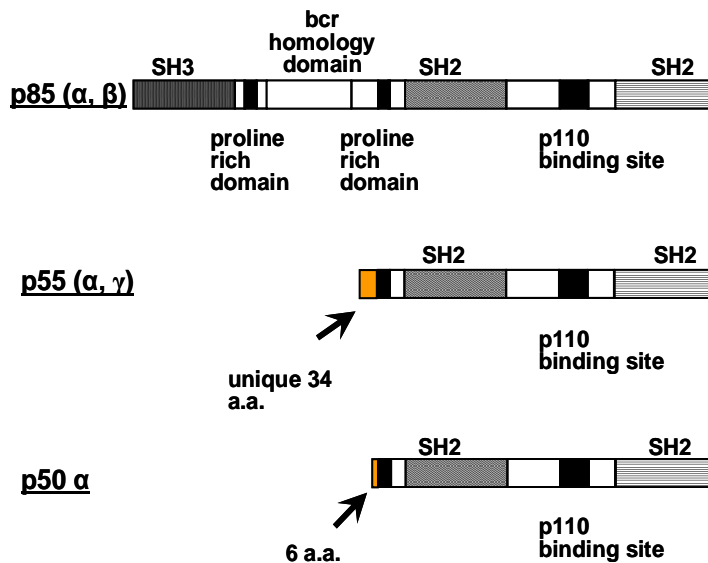


Figure 4. Schematic representation of different regulatory subunits of Class IA PI-3 Kinase. Class IA PI-3K comprises five different regulatory subunits encoded by three different genes. All regulatory subunits share two SH2 domains and an inter-SH2 domain that binds to the p110 catalytic subunit. The amino terminal domains of SH3 and BH are unique for p85 α and p85 β regulatory subunits. Although the shorter regulatory subunits, p50 α , p55 α and p55 γ lack SH3 and BH domains, they carry a specific amino acid sequence at their amino terminal end.

Upon SCF stimulation, the p85 regulatory subunit of PI-3K binds to the phosphorylated 719 tyrosine residue of murine c-Kit receptor (Y721 of human c-Kit) in its kinase insert region (Rottapel, Reedijk et al. 1991; Lev, Givol et al. 1992). Mutation of tyrosine 719 of murine c-Kit to phenylalanine eliminates the capacity for p85 to associate with c-Kit but enhances SCF-induced increased PI-3K activity (Serve, Hsu et al. 1994). Association of the PI-3K complex with the activated c-Kit receptor via the p85 regulatory subunit allows conformational change and activation of p110 catalytic subunit. The activated PI3K complex then gets translocated to the membrane where its lipid substrate resides.

PI-3Ks activate phospholipid substrates by phosphorylating the D3 hydroxyl position of the inositol ring, which generates the active products phosphatidylinositol-(3) phosphate (PtdIns(3)P₃), phosphatidylinositol-(4,5)-bisphosphate (PtdInsP₂) and phosphatidylinositol-(3,4,5)-triphosphate (PtdInsP₃) (Hawkins, Anderson et al. 2006). These products of class I PI-3K are crucial secondary messengers that recruit proteins containing a pleckstrin homology (PH) domain. Recruitment to cellular membranes activates them and initiates signaling cascades to enhance mast cell migration, adhesion, activation, growth and survival (Serve, Yee et al. 1995; Vosseller, Stella et al. 1997).

VIII. SCF and enhanced mast cell survival

Stem cell factor is the ligand for c-Kit that is primarily produced by stromal cells. SCF is a critical regulator of mast cell survival and mast cell numbers in various tissues *in vivo*. SCF rescues mast cells from spontaneous apoptosis *in vitro* (Mekori, Oh et al. 1993; Iemura, Tsai et al. 1994; Finotto, Mekori et al. 1997). Inhibition of SCF synthesis *in vivo* leads to mast cell apoptosis (Mekori, Oh et al. 1993; Iemura, Tsai et al. 1994; Finotto, Mekori et al. 1997). SCF stimulation of mast cells increases the level of pro-survival

proteins including Bcl-2, Bcl-X_L (Mekori, Gilfillan et al. 2001; Baghestanian, Jordan et al. 2002), which are critical for mast cell survival. Another mechanism by which SCF enhances mast cell survival is by actively preventing Bim expression via phosphorylation of forkhead box (FOX) proteins FOXO1a and FOXO3a. Bim is involved in mast cell apoptosis induced by growth factor deprivation (Alfredsson, Puthalakath et al. 2005).

PI-3K, an immediate downstream effector of c-Kit, is a well-known mediator of anti-apoptotic signaling, and elevated levels of PI-3K products are reported in many tumor cells (Downward 2004). Use of inhibitor LY294002, which selectively blocks PI-3K activation in cells, induces apoptosis and suppresses growth of tumor cells (Krystal, Sulanke et al. 2002). Abolition of PI-3K activation by mutating its docking site to c-Kit (Y719F) in mast cells results in significantly reduced survival (Serve, Yee et al. 1995). SCF-mediated c-Kit signaling in normal cells activates AKT, a survival-promoting serine-threonine protein kinase that enhances cell survival by inducing phosphorylation and inactivation of the pro-apoptotic molecule Bad. The phosphorylation and activation of AKT is, in turn, mediated by PI-3K upon SCF stimulation (Blume-Jensen, Janknecht et al. 1998). Activation of PI-3K also promotes phosphorylation of Bim and its proteasome-dependent degradation, thus enhancing survival. Although PI-3K is known to be critical for mast cell survival, the specific roles of PI-3K regulatory subunits in mediating the process are poorly understood. Using genetic and molecular approaches, we examined the role of p85 α and p85 β in regulating mast cell survival in response to SCF stimulation.

IX. Role of IL-3 in mast cell maturation

Differentiation and maturation of mast cells involve the synthesis and storage of an array of inflammatory mediators including histamine, mast cell proteases (mainly tryptases and chymases) and cytokines. IL-3 has been identified as one of the principle cytokines

regulating mast cell growth and terminal differentiation (Yong 1997; Galli, Nakae et al. 2005). *In vitro* cultures of murine bone marrow cells differentiate into a population of homogeneous mast cells when cultured in a medium supplemented with IL-3 (Razin, Ihle et al. 1984; Thompson, Metcalfe et al. 1990). IL-3 signaling activates Stat5, a transcription factor that is critical for mast cell development, as shown by the loss of mast cells in *Stat5*-deficient mice (Shelburne, McCoy et al. 2003). IL-3 also stimulates TNF secretion induced via the PI-3K pathway, which is crucial for the maturation of mast cells from their progenitors (Wright, Bailey et al. 2006).

In this thesis study, we characterized the specific role of p85 α and p85 β in regulating mast cell maturation *in vitro* in response to IL-3 stimulation. We further performed reconstitution studies to examine the role of the SH3 and BH domains of p85 α and p85 β in mast cell maturation. Furthermore, we confirmed our *in vitro* observations *in vivo*, by performing transplant studies.

X. Role of transcription factors in mast cell maturation

Transcription factors play a crucial role in the development of various lineages from uncommitted precursor cells. According to the current models for hematopoietic development, uncommitted stem cells express low levels of transcription factors (Orkin 2000; Cantor and Orkin 2001; Orkin 2003). Differentiation of mature blood cell types from the multipotential hematopoietic cell is controlled in part through the expression of lineage-specific transcription factors that regulate the expression of downstream genes that determine the function of each blood cell type (Iwasaki, Mizuno et al. 2006). The transcription factors GATA-1, GATA-2, PU.1, and the microphthalmia-associated transcription factor (Mitf) play essential roles in mast cell development. GATA-1, GATA-2, and PU.1 transcription factors are involved in the maturation of mast cells, while Mitf is

involved in the migration, phenotypic expression and survival of mast cells (Kitamura, Oboki et al. 2006).

A. GATA

GATA-1 is a member of highly conserved family of zinc finger protein that regulates the expression of several mast cell target genes, including FcεRI (Nishiyama, Hasegawa et al. 2002; Maeda, Nishiyama et al. 2003), carboxypeptidases (Zon, Gurish et al. 1991), IL-4 (Henkel and Brown 1994) and IL-13 (Masuda, Yoshikai et al. 2004). GATA-1^{low} mice generated by targeted deletion of upstream enhancer and promoter sequences of the GATA-1 gene show impaired differentiation of mast cells (Migliaccio, Rana et al. 2003). Mast cell defects associated with decreased GATA-1 expression was characterized by hyperproliferation of early mast cell precursors, susceptibility to apoptosis and impaired expression of FcεRI and mast-cell-specific carboxypeptidase-A in mature mast cells. (Migliaccio, Rana et al. 2003). Abberant insertion of a neomycin cassette between GATA-1 promoter regulatory elements and the GATA-1 IE exon also resulted in low GATA-1 expression (Takahashi, 1997). These mutant mice had lower GATA-1 levels and low populations of mature mast cells in the skin (Harigae, Takahashi et al. 1998). The increased apoptotic rate and defective differentiation of mast cells in GATA-1^{low} mice were reversed *in vitro* by forced GATA-1 expression (Migliaccio, Rana et al. 2003).

GATA-2 is another member of the GATA family that plays a crucial role in mast cell development. A GATA-2 deficiency in embryonic stem cells impaired the response to the c-Kit ligand SCF. Furthermore, those cells are incapable of differentiating into MCps (Tsai, Keller et al. 1994). Expression of GATA-2 is highest in proliferating mast cell lineage cells and is downregulated in differentiated mast cells (Jippo, Mizuno et al. 1996).

B. PU.1

Ets family member PU.1 is another transcription factor required for normal development of mast cells. PU.1-deficient murine fetuses lack dermal mast cells; PU.1 in cooperation with GATA regulates expression of crucial mast cell genes including FcεRI and IL-4 (Henkel and Brown 1994; Nishiyama, Hasegawa et al. 2002). In addition, evidence exists regarding an interplay between PU.1 and GATA in the regulation of mast cell development.

C. Mitf

Mitf (Microphthalmia transcription factor) is a basic helix-loop-helix leucine zipper transcription factor that regulates transcription of several genes essential for the growth, maturation, differentiation and normal histochemical composition of mast cells. Mitf directly targets protease genes including, mast cell proteases 2,4,8,9, granzyme, tryptophan hydroxylase, protease inhibitor Serpin E1, and metabolic enzyme hPGDs, and thus play a key role in mast cell biology (Morii, Tsujimura et al. 1996; Ito, Morii et al. 1998; Morii, Oboki et al. 2004)

Mitf is crucial of mast cell survival, and is expressed in mast cells as well as in the tissues where mast cells develop. Cultured mast cells derived from Mitf-mutant mice adhere poorly to fibroblasts. This defective adhesion has been attributed to deficient transcription of adhesion factor SgIGSF (Morii, Oboki et al. 2004), the factor also responsible for reduced peritoneal mast cell expression in Mitf mutants (Morii, Oboki et al. 2004). Mitf regulates c-Kit expression in mast cells; and, in turn, c-Kit regulates the transcriptional activity of Mitf, suggesting the possibility of homeostatic regulation between these factors (Isozaki, Tsujimura et al. 1994). Interestingly, a phenotypic overlap is seen among mice carrying a germ line mutation in the locus that encodes

SCF, receptor c-Kit, PI-3K and Mitf transcription factor (Dubreuil, Forrester et al. 1991; Moore 1995; Hemesath, Price et al. 1998; Fukao, Yamada et al. 2002).

In our study, we observed defective differentiation of mast cells in the absence of the p85 α regulatory subunit of PI-3K, which could be corrected upon restoring expression of either full-length p85 α or mutant constructs of p85 α that lack either amino terminal SH3 or BH domain. Although Mitf is critical in mast cell differentiation, the molecular mechanism regulating the expression of Mitf in mast cells is not well understood. Here we investigated the role of the p85 α regulatory subunit and its amino terminal domains in mediating Mitf expression in mast cells, which thereby regulates their maturation.

XI. *In vivo* experiments to determine the function of p85 regulatory subunits and their amino terminal domains in mast cell development

c-Kit and its ligand SCF are critical for the biological functions of mast cells and mast cell development is disrupted or severely affected in mice that either lack c-Kit expression or carry c-Kit mutations that result in its loss of function. Such mast-cell-deficient rodents are widely used as *in vivo* tools to investigate mast cell biology. One c-Kit mutation resulting in loss of function is *W-Sash* (W^{sh}), an inversion mutation upstream of c-Kit's transcriptional site on murine chromosome 5 (Berrozpe, Timokhina et al. 1999). This mutation specifically impairs the development of mast cells and melanocytes, and *in vitro* cultures from those mice do not express c-Kit mRNA (Duttlinger, Manova et al. 1993; Yamazaki, Tsujimura et al. 1994). Our study sought to investigate the role of p85 regulatory subunits in mediating mast cell development using an *in vivo* W^{sh} model. To determine the specific roles of p85 regulatory subunits and their amino terminal domains (SH3 and BH) in signal transduction of c-Kit/SCF mediated mast cell development, $W^{sh/sh}$ mice were transplanted with p85 α -deficient BMMC reconstituted with p85 α , p85 β ,

p85 α Δ SH3 and p85 α Δ BH constructs, and the mast cell population reconstituted in various tissues was investigated.

XII. Focus of the dissertation

Previous studies revealed the importance of PI-3K in mediating normal and abnormal c-Kit signaling, thereby regulating growth, differentiation, and survival mast cell biology. PI-3K directly interacts with c-Kit via its regulatory subunit; five different regulatory subunits have been reported (Inukai, Funaki et al. 1997). We hypothesize that p85 α and p85 β regulatory subunits might have unique mast cell functions that might be imparted by their amino terminal SH3 and BH domains, regions where the p85 subunits differ the most. We sought to investigate the specific roles of p85 α and p85 β , and the amino terminal SH3 and BH domains of p85 α in mediating mast cell growth, differentiation and survival. PI-3K might also enhance c-Kit receptor internalization and degradation via binding to the activated ubiquitin ligase, c-Cbl. We therefore investigated the interaction of p85 regulatory subunits with c-Cbl, and their role in regulating the c-Kit internalization and degradation process upon SCF stimulation. Information on specific roles of PI-3K regulatory subunits and their domains in regulating c-Kit signals in mast cells advances our understanding of the mechanisms critical to the biology of mast cell growth and differentiation for future translational applications for the treatment of mast cell disorders.

MATERIALS AND METHODS

I. Cytokines, Antibodies and Reagents

Recombinant murine interleukin-3 (IL-3), stem cell factor (SCF), interleukin-6 (IL-6), Flt3 and Thrombopoietin (TPO) were purchased from Pepro Tech, Rocky Hill, NJ. Phycoerythrin (PE)-conjugated c-Kit antibody, fluorescence isothiocyanate (FITC)-conjugated IgE receptor antibody, PE-conjugated Annexin V antibody, 7-Amino actinomycin D (7-AAD), and allophycocyanin (APC)-conjugated c-Kit antibody were purchased from BD Biosciences, San Jose, CA; and PE-conjugated anti-mouse Fc ϵ R1 α antibody was purchased from eBioscience Inc, San Diego, CA. Rabbit anti-c-Kit receptor antibody (C-19) was purchased from Santa Cruz Biotechnology, Santa Cruz, CA. Mouse anti-hemagglutinin (anti-HA) antibody, rabbit anti-p85 pan antibody (clone UB93-3), mouse anti-p85 α -specific antibody (clone AB6) and rabbit phosphotyrosine antibody were purchased from Upstate Biotechnology Inc., Lake Placid, NY. Rabbit anti-phospho-AKT (193H12), anti-phospho-ERK (197G2), Gab1, Gab2 (26B6), Grb2, mouse-anti ERK (clone M12), rabbit anti AKT and anti-Ubiquitin (P4D1) antibodies were acquired from Cell signaling Technology, Beverly, MA, phospho-c-Cbl antibody was purchased from Epitomics Inc, Burlingame, CA, and anti-p110 α antibody (RB1700) was obtained from Abgent Inc, San Diego CA. Anti-p110 δ antibody (Clone 29) and anti-SHIP antibody (Clone 32) was purchased from BD Biosciences, San Jose, CA, mouse anti-Mitf antibody was generated by Dr. Clifford Takemoto, Protein A- or Protein G-sepharose beads were purchased from Amersham Biosciences, Piscataway, NJ. Retronectin was purchased from Takara, Madison, WI. Iscove's Modified Dulbecco's Medium (IMDM) was purchased from Invitrogen, Carlsbad, CA, and [3 H] thymidine was purchased from PerkinElmer, Boston, MA. Antibody dilutions were followed as per manufacturer's protocol.

II. Mice

C57BL/6 mice and C3H/HeJ mice were purchased from Jackson Laboratory (Bar Harbor, ME). *p85α*^{-/-} mice have been previously described (Terauchi, Tsuji et al. 1999). Generation of mice lacking p85β (*p85β*^{-/-}) with disruption of the first exon of the *Pik3r2* gene by homologous recombination has been described previously (Ueki, Yballe et al. 2002). Mast cell deficient *W^{sh}* mice have been previously described (Duttlinger, Manova et al. 1993). All mice were maintained under specific pathogen-free conditions at the Indiana University Laboratory Animal Research Center (Indianapolis, IN).

III. Cell lines

The murine IL-3 dependent myeloid cell line 32D cells were used to express c-Kit and p85 regulatory subunits of PI-3 kinase. These cells were cultured in IMDM supplemented with 10% fetal bovine serum and IL-3 (10 ng/ml).

IV. Cloning

A. Construction of the HA tagged-full length p85 constructs

Mouse full-length p85α and p85β cDNA were inserted into a MIEG3 vector, which encoded an enhanced green fluorescence (EGFP) protein creating GFP-tagged p85 constructs. These p85α constructs also carry an HA tag at its amino terminus.

i. p85α

After synthesizing cDNA, the following primers were used for amplification of p85α by PCR: forward, 5'-GAATTCATGTACCCATACGATGTTCCAGATTAC GCTATGAGTGCAGAGGGCTACCAG; reverse, 5'-CTCGAGTCATCGCCTCTG TTGTGCATATAC. Restriction sites used for cloning purposes are underlined.

ii. p85 β

A pGEX-4T3 plasmid containing murine full-length p85 β gene was purchased from Addgene, Cambridge, MA, and the full-length p85 β was amplified using primers: forward, 5'-CAAGAATTCATGTACCCATACGATGTTCCAGATTACGCTGCAGGAGCC GAG and reverse, 5'-CACCTCGAGTCAGCGTGCT GCAGACGG. (Restriction sites are underlined.)

HA-tagged constructs were cloned to the *EcoRI/XhoI* site upstream of an internal entry site of bicistronic retroviral vector MIEG3.

B. Construction of the HA-tagged p50 α construct

The shorter isoform p50 α was cloned from p85 α using primers: forward, 5'-AGAATTCATGTACCCATACGATGTTCCAGATTACGCTCATAACCTGCAAACACTGCC CCCC and reverse 5'-CTCGAGTCATCGCCTCTGTTGTGC. The forward primer binds to amino acid residues 307-311 of p85 α , and the reverse primer binds to amino acid residues 719-724 of p85 α . The PCR product was subjected to restriction digestion with *EcoRI* and *XhoI* enzymes, and was cloned to the MIEG3 vector to create GFP-tagged p50 α construct. The construct also carries an HA tag at its amino terminus.

C. Construction of the HA-tagged p85 mutant constructs

For cloning of p85 α with a deletion of either SH3 domain (i.e., p85 α Δ SH3; amino acid residues 81–724 of p85 α), or BH domain (i.e., p85 α Δ BH; amino acid residues 1–101 and 289–724 of p85 α), the full-length version of p85 α was used as a template.

i. p85 α Δ SH3

For the amplification of p85 α Δ SH3 from p85 α , we used the primers: forward, 5'-CCAGAATTCATGTACCCATACGATGTTCCAGATTACGCTAGAATTTACCCCCCTACTCCC and reverse: 5'-CCACTCGAGTCATCGCCTCTGTTGTGCAT ATACTGG. (Restriction sites are underlined.) The forward primer binds to amino acids 81–83, and reverse primers binds to 716–724 amino acids of the *p85 α* gene.

ii. p85 α Δ BH

We used two sets of primer to clone the p85 α Δ BH construct from p85 α . To amplify the amino acid region 1–101 of p85 α , we used the primers: forward, 5'-CAAGAATTCATGTACCCATACGATGTTCCAGATTACGCTAGTGCAGAGGGCTAC and reverse: 5'-CTCTATCGCAGAACCCGGAGCAACAGGAAGC GGTCG. The forward primer binds to 2–5 amino acid residues of the p85 α template, and the reverse primer binds to 95–101 amino acid residue of p85 α .

A second set of primer: forward, 5'-CCGGGTTCTGCGATAGAGATTTTAATC and reverse, 5'-CACCTCGAGTCATCGCCTCTGTTGTGC, was used to amplify the amino acid region 289–724 of p85 α . The forward primer binds to 289-291 amino acid residues and the reverse primer binds to 719–724 amino acid residues of p85 α .

The two amplified products were linked together by another PCR using primers: forward, 5'-CAAGAATTCATTACCCATACGATGTTCCAGATTACGCTAGTGCAGAGGGCTAC and reverse, 5'-CACCTCGAGTCATCGCCTCTGTTGTGC (restriction sites are underlined) to construct p85 α Δ BH.

Amino terminal HA-tagged mutant p85 α constructs were then cloned to the *EcoRI/XhoI* site of the bicistronic retroviral vector MIEG3.

D. Construction of the HA-tagged p85 chimeric constructs

Chimeric constructs, p85 $\alpha\beta$ (which has the amino terminal SH3 and BH domain of p85 α , and carboxy terminal containing two SH2 and inter SH2 domain of p85 β) and p85 $\beta\alpha$ (which has the amino terminal SH3 and BH domains of p85 β , and carboxy terminal containing two SH2 and inter SH2 domain of p85 α) were cloned using the full-length version of p85 α and the full-length version of p85 β as templates

i. p85 $\alpha\beta$

For construction of p85 $\alpha\beta$, the portion of the p85 α encoding the amino terminal half of the protein was as a template and amplified by PCR using a full-length p85 α cDNA as template and primers: forward, 5'-CAAGAAATTCATGTACCCATACCCATACGAT GTTCCAGATTACGCTAGTGCAGAG and reverse, 5'-CCCCAGTACCATTTCAGC. Here, the forward primer binds to codons 2–4 of p85 α and reverse binds to codons 331–334 of p85 α . The carboxy terminal half of p85 β was amplified using a full-length p85 β cDNA as a template and amplified using primers: forward, 5'-TGGTACTGGGGGGACATC and reverse, 5'-CAACTCGAGTCAGCGTGCTGCAG. The forward primer binds to codons 326–329 of p85 β , and the reverse binds to codons 718–722 of p85 β cDNA template. The two products were joined together by performing another PCR using these amplified products and primers: forward, 5'-CAAGAAATTCATGTACCCATACCCATAC GATGTTCCAGATTACGCTAGTGCAGAG and reverse, 5'-CAACTCGAGTCAGCGTG CTGCAG. (Restriction sites are underlined.)

ii. p85 $\beta\alpha$

Similarly, for construction of p85 $\beta\alpha$ clone, the p85 β cDNA was used as a template to amplify the portion encoding the N-terminal portion of the protein using the primers: forward, 5'-CAAGAATTCATGTACCCATACGATGTTCCAGATTACGCTGCAGGAGCCCGA and reverse: 5'-GTCTCCCCAGTACCACTCTGCATCCTGAAGC and full length p85 β as the template. The forward primer binds to codons 2–5, and reverse primer binds to codons 319–325 of the p85 β cDNA. The portion of the p85 α encoding the carboxy terminal half was amplified using the full-length p85 α cDNA template and primers, forward: 5'-GAGTGGTACTGGGGAGACATCTCAAGG and reverse: 5'-CACCTCGAGTCATCGCCTCTGTTGTGC. The forward primer binds to codons 335–340, and the reverse binds to codons 719–724 of p85 α . The two amplified products were linked together by performing another PCR using primers: forward, 5'-CAAGAATTCATGTACCCATACGATGTTCCAGATTACGCTGCAGGAGCCCGA and reverse, 5'-CACCTCGAGTCATCGCCTCTGTTGTGC.

Amino terminal HA-tagged chimeric p85 constructs were then cloned to the *EcoRI/XhoI* site of the bicistronic retroviral vector MIEG3. PCR was performed using the following conditions: an initial denaturation step at 94°C for 2 min, followed by 23 cycles at 94°C for 30 s, 60°C for 1 min, and 72°C for 2 min, with a final step at 72°C for 7 min. All the constructs having HA tag at the amino termini were cloned to the *EcoRI/XhoI* site upstream of an internal entry site and the enhanced green fluorescence (EGFP) protein containing the bicistronic retroviral vector MIEG3.

V. Preparation of retroviral supernatants for transduction

Retroviral supernatants for transduction of 32D cells and primary bone marrow cells were generated using the Phoenix ecotropic packaging cell line transfected with

retroviral vector plasmids using a calcium phosphate transfection kit. Phoenix GP cells were cultured in gelatin coated tissue culture plates in Dulbecco's Modified Eagle Medium (DMEM high glucose media), supplemented with 10% FBS, 2% penicillin/streptomycin and 1% glutamine. 70% confluent GP cells were transfected to generate retroviral supernatants for various constructs. For transfection, 15 µg of MIEG3 plasmid was used, in which we cloned various constructs of p85; 10 µg of gag-pol plasmid; 3 µg of envelope plasmid; 36 µl of 2M calcium chloride. The reaction volume was adjusted to 300 µl by adding ddH₂O. To this reaction mixture, 300 µl of 2xHBS buffer was added, and the contents were mixed thoroughly by bubbling air for 30 s. The reaction mixture was then incubated at room temperature for 30 min, after which, 9 ml of growth media (DMEM containing 10% FBS, 2% Penstrep, 1% Hepes buffer solution and 1% glutamine) was added. The pH of the growth media was adjusted to 7.9 before the reaction mixture was added to each GP plate. On the first day of infection, 9 µl of chloroquine (20mM) was also added to the plates. Forty-eight hours post transfection, viral supernatants were collected from these plates after filtering them through 0.45 µM membranes.

VI. Generation of mast cells

Low-density bone marrow cells (LDBM) were harvested from wild-type (WT) and knockout mice. Bone marrow cells were flushed with prewarmed IMDM using a 23¹/₂ G needle. Bone marrow cells were suspended in 5 ml IMDM and were subjected to ficol gradient separation by spinning at 1500 RPM at 25°C in spin cycles without breaks. After washing out leftover ficol, cells were cultured in IMDM supplemented with 10% fetal bovine serum and IL-3 (10 ng/ml). These cells were used to generate mast cells or to do retroviral transduction and transplantation studies.

VII. Expression of p85 constructs in 32D cells and Mast Cell Progenitors (MCps)

To express different PI-3K regulatory subunits in 32D cells, cells were infected with 2 ml of high-titer retroviral supernatants in the presence of 8 µg/ml polybrene and 10 ng/ml IL-3 for 48 h. To express different regulatory subunits in mast cell progenitors, low-density bone marrow cells were collected from WT and *p85α^{-/-}* mice, and pre-stimulated in IMDM supplemented with 20% FBS, 1% penicillin/streptomycin, and cytokines (100 ng/ml SCF and 10 ng/ml IL-3) for 2 days, prior to retroviral infection on fibronectin fragment (Retronectin) in non-tissue culture plates. Three days after harvesting, MCps were transfected with high-titer retroviral supernatants for the indicated constructs. Retronectin-coated non-tissue culture 6-well plates were preloaded with the retrovirus by spinning them with 4ml viral supernatants at 1500 RPM at 25°C for 30 min. After centrifugation, viral supernatants were aspirated, and the plates were loaded with 4 ml fresh viral supernatants from the same constructs, along with 2×10^6 cells from WT or *p85α^{-/-}* mice. These plates were subjected to another round of spinning at 1500 RPM for 30 min at 25°C, 800 µl of FBS, 10 ng/ml IL-3 and 100 ng/ml SCF were added to each well and incubated at 37°C. The next day, cells were subjected to another round of retroviral infection. On the following day, cells were harvested and resuspended in IMDM supplemented with 10% FBS and 10 ng/ml of IL-3. The cells expressing EGFP were sorted and further studied.

VIII. Transplant studies

For the mast cell reconstitution study *in vivo*, *p85α^{-/-}* LDBM cells were transduced with various constructs of p85α (p85α, p85β, p85αΔSH3, p85αΔBH) and empty vectors were transplanted to *W^{sh/sh}* mice. This transplantation was also carried out using fluorouracil (5-FU)-treated LDBM cells.

For LDBM transplants, 10–12 wk old *p85 α ^{-/-}* mice were sacrificed; femur, tibia and iliac bones were harvested and flushed with prewarmed IMDM using a 23¹/₂ G needle. Bone marrows were subjected to ficol gradient separation, and LDBM were prestimulated for 48 h at 37°C in non-tissue culture plates with a cytokine cocktail: 10 ng/ml interleukin-6, 100 ng/ml stem cell factor, 50 ng/ml Flt-3 ligand, and 100 ng/ml thrombopoietin in IMDM supplemented with 20% FBS. On Day 3 after harvest, LDBM cells were transfected with 4 ml of high-titer retroviral supernatants for various constructs (p85 α , p85 β , p85 α Δ SH3, p85 α Δ BH and empty MIEG3 vector). A second shot of viral infection was given 24 h later. Forty-eight hours after the first infection, cells were collected and grown in IMDM supplemented with 10% FBS and cytokines (10 ng/ml interleukin-6, 100 ng/ml stem cell factor, 50 ng/ml Flt-3 ligand, and 100 ng/ml thrombopoietin). The following day, enhanced green fluorescent protein (EGFP)-expressing cells were purified by fluorescence-activated cell sorting (FACS).

For 5-FU transplant studies, a single dose of 5-FU (150 mg/kg body weight) was injected intra peritoneally into *p85 α ^{-/-}* mice. 3 days after injection, low-density bone marrow cells were isolated as described above. LDBM cells were pre-stimulated in IMDM supplemented with 20% fetal bovine serum and murine cytokines (10 ng/ml interleukin-6, 100 ng/ml stem cell factor, 50 ng/ml Flt-3 ligand, and 100 ng/ml thrombopoietin) for 2 days. Prestimulated cells were transduced with a retrovirus encoding an empty MIEG3 vector, full-length p85 α , full-length p85 β , p85 α Δ SH3 or p85 α Δ BH. After 2 rounds of infection, EGFP-expressing cells were purified by FACS.

For mast cell reconstitution study with LDBM or (5-FU)-treated LDBM cells, 1×10^6 transduced; and sorted cells were mixed with 1×10^5 supporting fresh splenocytes from

$W^{sh/sh}$ mice and administrated intravenously by tail-vein injection into lethally irradiated $W^{sh/sh}$ recipient mice (1100 cGy split dose with 4 h interval).

IX. Immunoprecipitation

Immunoprecipitation (IP) was performed using lysates prepared from 32D cells expressing c-Kit and either full-length or mutant p85 constructs. 32D cells transduced with various constructs were cultured in IMDM supplemented with 10% fetal bovine serum and 10 ng/ml IL-3. Cells were starved for serum and then treated with SCF (100 ng/ml) to visualize the specific protein interaction in response to stimulation of c-Kit with ligand SCF. For starvation, cells in culture were pelleted down, washed twice with IMDM to remove serum and cytokine, then resuspended in starvation media (IMDM supplemented with 0.2% BSA). 5×10^6 cells were resuspended in 10 ml starvation media and plated in petridish overnight. Starved and SCF stimulated cells were lysed in a lysis solution (10 mM K_2HPO_4 , 1 mM EDTA, 50 mM EGTA, 10 mM $MgCl_2$, 1 mM Na_2VO_4 and 50 mM β -glycerol phosphate) supplemented with protease inhibitors (10 μ g/ml aprotinin, 10 μ g/ml leupeptin, 1 μ g/ml pepstatin A [pH 7.2]). Lysates were then clarified by centrifugation at $10,000 \times g$ for 30 min at 4°C. Protein content in the lysates was determined by the BCA assay (Thermo Fisher Scientific Inc., Rockford, IL).

The immunoprecipitation assays were carried out as previously described (Kapur, Majumdar et al. 1998). Briefly, 100 μ g of cell lysate was incubated with 10 μ l anti-c-Kit receptor antibody or anti-hemagglutinin (anti-HA) antibody overnight at 4°C. Protein-A and Protein-G Sepharose beads (GE Healthcare) were washed 3 times with ice-cold PBS to remove any ethanol, and then resuspended with one bed volume of PBS. 25 μ l of beads suspended in PBS were added to each microfuge tube and incubated for 4 h at 4°C to insure that the protein bound to is complexed to beads. The immunoprecipitates

recovered with the beads were washed twice with 1 ml ice-cold PBS and one time with 1ml 1x cell lysis buffer to remove any nonspecific binding, and then boiled with 60 µl of 1x sample buffer. The bound proteins were then subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) followed by and immunoblotting using the indicated antibodies.

X. Immunoblotting

Equal amounts of the protein lysates were fractionated by electrophoresis using a 10% SDS-polyacrylamide gel and then electrophoretically transferred onto a nitrocellulose membrane. The nitrocellulose membrane was first incubated with a blocking solution consisting of 5% nonfat dry milk in Tris-buffered saline containing 0.1% Tween-20 (TBS-T). Membranes were then probed the indicated antibodies (1:2000 dilutions) at 4°C overnight using rocker for gentle agitation of the solutions. The membranes were washed three times with TBS-T for 10 min, followed by incubation with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies (1:3000 dilution) for 1 h at room temperature. Then membranes were again washed three times with the TBS-T solution. Finally, the targeted proteins on the membranes were detected with SuperSignal West Dura luminol/enhancer solution and the membranes were exposed to X-ray film.

XI. Proliferation assay

Cell proliferation was assessed by conducting a thymidine incorporation assay as described (Vemula, Ramdas et al.). *In vitro* mast cells generated from different mice and EGFP-sorted *in vitro*-generated MCps expressing either empty vector or various constructs of p85 were measured for proliferation. Briefly, cells were washed twice with warm IMDM and starved in IMDM supplemented with 0.2% BSA for 6 to 7 h. 5×10^4

cells were plated in a 96-well plate in 200 μ l of IMDM supplemented with 10% fetal bovine serum plus 2% penicillin/streptomycin in the presence or absence of SCF. Cells were cultured for 48 h and subsequently pulsed with 1.0 μ Ci of [3 H] thymidine for 6 to 8 h at 37°C. Cells were harvested using an automated 9-well cell harvester (Brandel; Gaithersburg, MD) and thymidine incorporation was determined as counts per minute (CPM).

XII. Apoptosis and Cell Cycle

Low-density bone marrow cells harvested from WT and knockout mice were cultured in IMDM supplemented with 10% fetal bovine serum and 10 ng/ml IL-3. To determine the cell cycle and measure apoptosis in these cells in response to SCF stimulation, cells were collected by centrifugation, and then washed 2 times with IMDM to remove any serum or cytokines. The cells were then starved for 7 h in IMDM supplemented with 0.2% BSA. After this, the number of cells were determined, and 0.2×10^6 cells were resuspended in IMDM supplemented with 10% FBS and plated into each well of a 24-well tissue culture plate. For stimulated conditions, we supplemented the wells with 100 ng/ml SCF, while unstimulated wells were deprived of any cytokines. After 48 h, the levels of apoptosis were determined by first incubating the cells in 100 μ l of 1x annexin binding buffer for 20 min at 4°C, after which the cells were stained with 1 μ g 7-AAD and PE-Conjugated Annexin-V antibody and analyzed by flow cytometry. For cell-cycle analysis, starved and stimulated or unstimulated (2×10^6) cells were labeled by propidium iodide (PI)/RNase and analyzed by flow cytometry.

XIII. Sample preparation for microarray analysis

Low-density bone marrow cells were harvested from 3 independent WT and *p85 α ^{-/-}* mice and cultured in IMDM supplemented with 10% fetal bovine serum, 2%

penicillin/streptomycin, and 10 ng/ml of IL-3 for 1 week. Cells were collected by centrifugation, resuspended in 200 μ l PBS containing 0.2% BSA, and then incubated with 5 μ g of anti-PE- c-Kit and anti-FITC-IgE antibody for 30 min at 4°C. Cells were washed with a PBS solution containing 0.2% BSA to remove any unbound antibodies. The mast cell population that demonstrated double expression of c-Kit and IgE receptor was then sorted and collected. The sorted cells were then stimulated with SCF for 48 h, after which, they were pelleted, frozen immediately and stored at -80°C until use for microarray analysis.

XIV. Microarray processing and data analysis

Frozen cell pellets collected from 3 independent biological samples of WT and *p85 α ^{-/-}* mice were sent to Miltenyi Biotech to carry out the microarray analysis. Because our sample size was significantly small (<10,000 cells), super-amplification technology was required for the analysis. This amplification was based on a global PCR protocol using mRNA-derived cDNA. mRNA was isolated via magnetic bead technology. Amplified cDNA from the mRNA samples was quantified, and quality was evaluated by capillary electrophoresis on an Agilent 2100 Bioanalyzer platform (Agilent Technologies; Santa Clara, CA). 250 ng of WT and *p85 α ^{-/-}* cDNAs was labeled with fluorescent dye cy3 or cy5 and simultaneously hybridized overnight (17 h, 65°C) to a Agilent whole-mouse genome oligo microarray 4 \times 44K. In order to avoid differential incorporation of the 2 dyes into target samples, dye incorporation rate was carefully monitored. To avoid breakdown of Cy5 by ozone, an ozone-free microarray facility in Miltenyi Biotech was maintained with specially installed ozone extractors. Subsequently, microarrays were washed and signals were detected using Agilent's DNA microarray scanner. Probe-set intensities were obtained and a quantile normalization procedure was used to adjust for

the difference in probe intensity distribution across different chips. For our analysis, we included genes that showed a fold-change >1.5 and $p < 0.05$.

XV. c-Kit internalization experiment

32D cells co-transduced with c-Kit and p85 constructs (p85 α or p85 β) were first sorted for the cells that were double-positive for the expression of c-Kit and p85 construct. The surface expression of c-Kit in cells was detected by staining the cells with c-Kit biotinylated antibody, and the p85 constructs that were cloned into the MIEG3 vector were detected by green fluorescence. Sorted cells were grown in IMDM media supplemented with 10% fetal bovine serum and IL-3 (10 ng/ml). For our internalization experiment, sorted 32D cells were starved overnight in IMDM supplemented with 0.2% BSA and then treated with cyclohexamide (50 ng/ml) for 30 min at 37°C. Cells were then stimulated with SCF (100 ng/ml) at 37°C for 0, 5, 10 and 30 min, as indicated, and subjected to c-Kit staining. For this analysis, we added 1 μ g anti-PE c-Kit antibody to the cells in a 100 μ l of PBS and 0.2% BSA, and then incubated the mixture for 30 min at 4°C. To remove any unbound antibody, cells were then washed with PBS containing 0.2% BSA and analyzed by FACS.

RESULTS

I. Mast cells express multiple regulatory subunits of class IA PI-3 kinase (PI-3K)

To determine the expression of various Class IA PI-3K regulatory subunits in mast cells, Western blot analysis was performed on *in vitro* bone-marrow–derived mast cells (BMMC) from WT, *p85α*^{-/-} and *p85β*^{-/-} mice using a pan-p85 antibody, p85α-specific antibody and p85β-specific antibody to differentiate the subunits of p85. As seen in Figure 5A, mast cells express multiple Class IA PI3-K regulatory subunits including p85α, p85β, p55α and p50α. Loss of p85α and p85β in *p85α*^{-/-} and *p85β*^{-/-} mast cells was confirmed by Western blotting (Figure 5B and 5C).

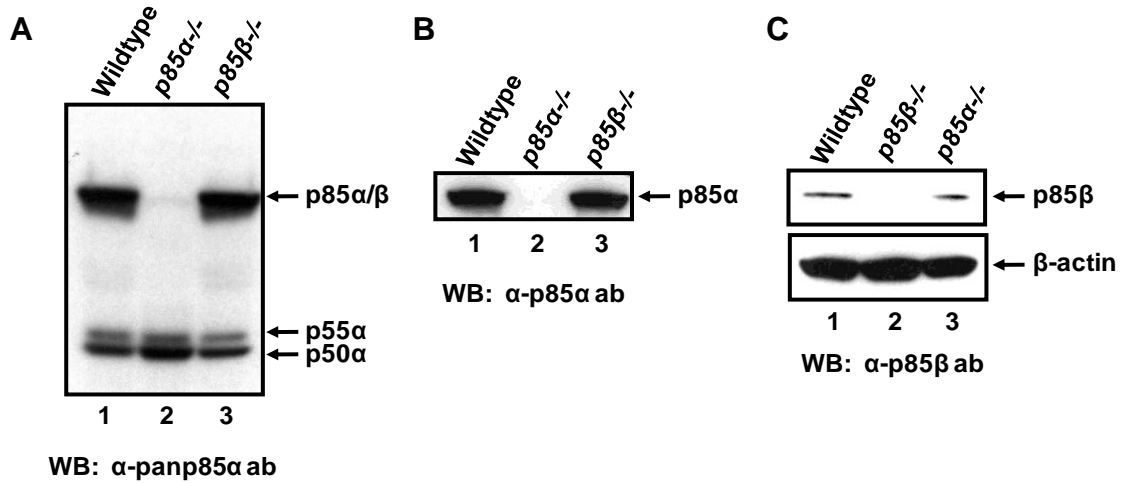


Figure 5. Expression of Class IA PI-3K regulatory subunits in WT, *p85α*^{-/-} and *p85β*^{-/-} bone-marrow-derived mast cells (BMMCs). BMMCs were harvested and equal amount of protein extracts were subjected to Western blot analysis using a pan-p85 antibody (recognizes all regulatory subunits of Class IA PI3-K), a p85α-specific antibody, a p85β-specific antibody, and a β-actin antibody as indicated. Expression of different PI-3K regulatory subunits is indicated. β-actin was used as a loading control for each lane (A–C).

II. The p85 α regulatory subunit of PI-3K is critical for biological functions of mast cells

A. Deficiency of p85 α in MCps results in defective growth of MCps in response to SCF stimulation

Various regulatory subunits of Class 1A PI-3K get activated upon binding to the 719 tyrosine residue of c-Kit upon SCF stimulation (Serve, Hsu et al. 1994). While mast cells express various regulatory subunits of Class 1A PI-3K (Figure 5A), the physiological roles of the various subunits in regulating c-Kit-induced mast cell growth and differentiation are not known. Here we sought to evaluate the specific roles of p85 α regulatory subunits in mast cell biology in response to SCF stimulation. To assess the contributions of p85 α to mast cell growth, the differences in proliferation was determined between mast cell progenitors (MCps) obtained from WT and p85 α ^{-/-} mice. MCps were starved of serum and cytokines for 6 h, after which they were cultured in the presence or absence of SCF for 48 h. These cells were then pulsed with [³H] thymidine for 6 h. Thymidine incorporation is a direct assessment of the proliferation rate of these cells in response to SCF stimulation. MCps from WT and p85 α ^{-/-} mice grown in the absence of growth factors showed minimal thymidine incorporation. As shown in Figure 6A, WT MCps demonstrated significant increase in thymidine incorporation in presence of SCF, whereas deficiency of p85 α resulted in complete loss of SCF mediated growth. This result suggests that the p85 α regulatory subunit is critical for SCF-induced mast cell growth in response to SCF stimulation.

B. Deficiency of p85 α results in defective maturation of MCps

We assessed how the loss of p85 α alters the maturation of MCps in response to IL-3. Earlier studies had demonstrated that media supplemented with the cytokine IL-3

facilitates the maturation of murine bone marrow cells to mast cells *in vitro* (Razin, Ihle et al. 1984). Low density bone marrow cells from WT and $p85\alpha^{-/-}$ mice cells were harvested and cultured in media supplemented with IL-3 (10 ng/ml). Maturation of these cells to mast cells was evaluated based on mast cell surface marker (c-Kit and IgE receptor) expression. Mast cell maturation was analyzed on weekly basis by staining MCps with antibodies to detect the expression of c-Kit and IgE receptors by flow cytometry. Figure 6B represents a dot blot profile of mast cell maturation at different points during development. While 91% of WT MCps fully matured into c-Kit and IgE receptor double-positive cells, $p85\alpha$ deficiency significantly reduced the maturation of MCps: only 20% of those cells matured into a mast cell population at the end of 4 weeks. The results suggest that $p85\alpha$ plays a crucial role in mast cell maturation as well.

C. Deficiency of $p85\alpha$ in MCps results in defective survival of MCps in response to SCF stimulation

Significantly reduced proliferation in $p85\alpha^{-/-}$ MCps might be due to an altered survival potential of these cells in the specific absence of $p85\alpha$. Here we evaluated the role of the $p85\alpha$ regulatory subunit in mediating mast cell survival after SCF stimulation. *In vitro* cultures of low-density bone marrow cells harvested from WT and $p85\alpha^{-/-}$ mice were starved for 6 h and grown in the presence or absence of SCF. After 48 h, apoptosis was assessed by staining the cells with antibodies to Annexin V and 7-AAD, followed by flow cytometry analysis. As seen in Figure 6C, less than 10% of MCps from WT and $p85\alpha^{-/-}$ mice survived for 48 h in the absence of any growth factors, as determined by the presence of cells negative for Annexin V and 7-AAD. Although WT MCps showed increased survival in the presence of SCF (50%), there was a significant reduction in the survival of $p85\alpha$ -deficient MCps (19%). These results suggest that $p85\alpha$ acts as a positive regulator of survival.

We then examined the cell cycle progression in WT and $p85\alpha^{-/-}$ MCps in response to SCF stimulation. Starved and SCF-stimulated (50 ng/ml) MCps were labeled with propidium iodide, and cell cycling was analyzed by flow cytometry. As shown in Figure 6D, SCF stimulation enhanced cell cycle progress in WT MCps, whereas cell cycle progression and S phase entry was significantly impaired in $p85\alpha^{-/-}$ MCps. The result suggests that p85 α plays a critical role in the cell cycle of MCps in response to SCF in mast cells.

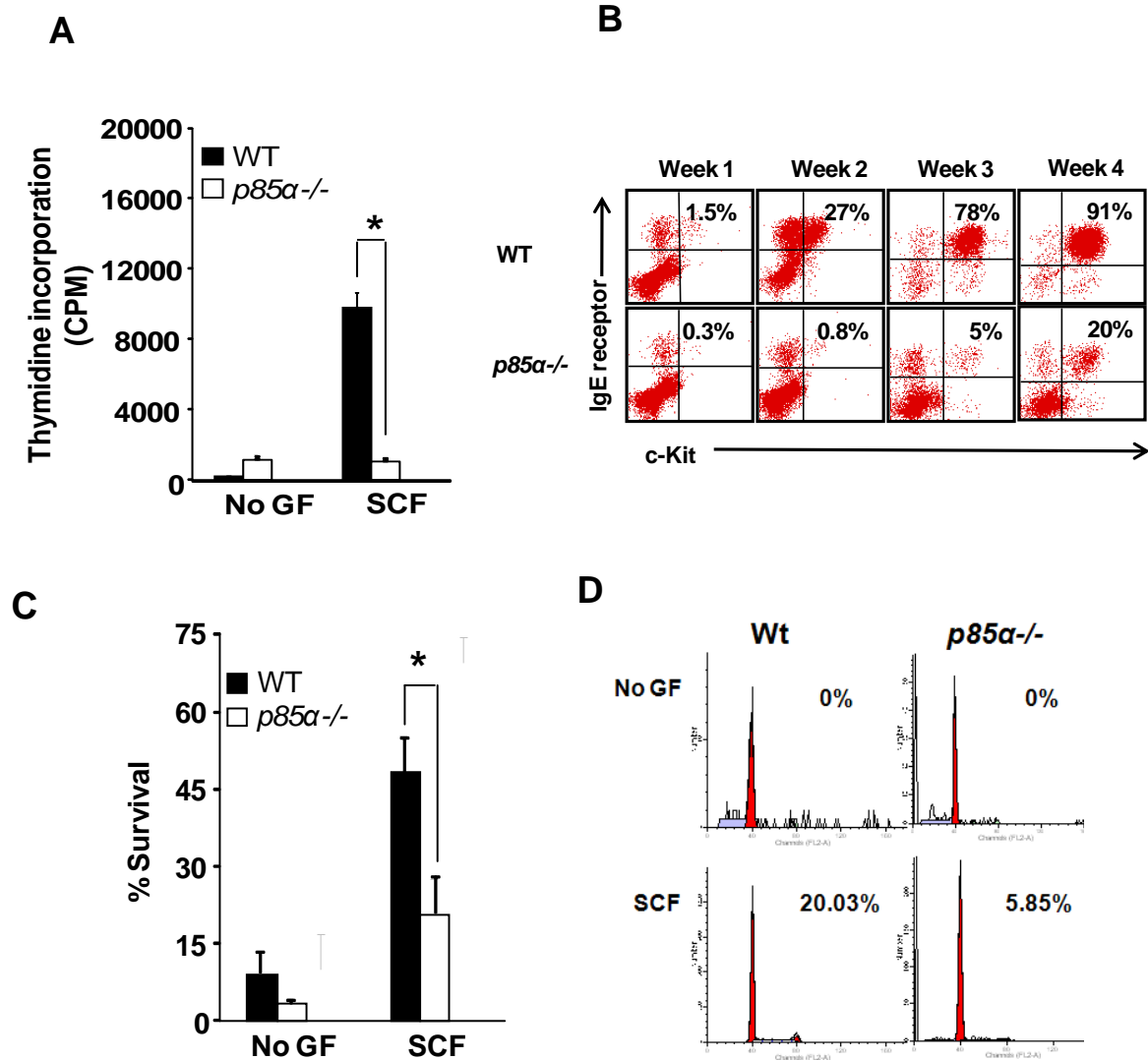


Figure 6. The *p85α* regulatory subunit of PI-3K is critical for biological functions of mast cells. (A) MCps from WT and *p85α*^{-/-} cells were starved for 6 h in serum- and cytokine-free media and cultured in the presence or absence of SCF (50 ng/ml). After 48 h, proliferation was evaluated by [³H] thymidine incorporation. The histograms represent the mean [³H] thymidine incorporation in MCps (CPM ± SD), with the results from one representative experiment performed in quadruplicate. Similar results were observed in six independent experiments. **p*<0.01, WT vs. *p85α*^{-/-}. (B) LDBMCs from WT and *p85α*^{-/-} mice were cultured in the presence of IL-3 (10 ng/ml) for total of 4 weeks. At indicated time points, maturation was analyzed by staining the cells with antibodies that recognize

c-Kit and IgE receptor followed by flow cytometry. Shown is dot blot profile from one of four independent experiments each resulting in the same conclusion **(C)** MCps from WT and *p85α*^{-/-} cells were starved for 6 h in serum- and cytokine-free media and cultured in the presence or absence of SCF (50 ng/ml). After 48 h, cells were stained with phycoerythrin-conjugated Annexin V and 7-AAD, followed by flow cytometry analysis. A representative dot blot showing the percent survival of MCps as determined by the lack of staining of cells by either Annexin V and/or 7-AAD (i.e., lower-left coordinate). **(D)** MCps from WT and *p85α*^{-/-} cells were starved for 6 h in serum- and cytokine-free media and cultured in the presence or absence of SCF (50 ng/ml). Cells were stained with propidium iodide and analyzed by flow cytometry. The percentage of cells in the S phase of the cell cycle is shown. Similar results were observed in three independent experiments; *p<0.05.

Although previously it was thought that all regulatory subunits of PI-3K functioned the same way, MCps lacking p85α regulatory subunit show severe defects in mast cell biological functions, irrespective of the presence of p85β, p50α and p55α (Figure 5). p85α and p85β, regulatory subunits, although encoded by two different genes, they share 100% protein domain homology and 60% amino acid identity (Figure 18). Noticably as seen in Figure 5, p85α is the most abundant PI-3K regulatory subunit expressed in MCps, and p85β is expressed in significantly lesser amounts.

III. p85 β does compensate for the loss of p85 α regulatory subunit in mast cell

To evaluate whether the defective mast cell phenotypes in *p85 α* ^{-/-} MCps, as well as; the inability of p85 β to compensate for the loss of p85 α is due to the quantitative reduction in overall expression of PI-3K regulatory subunits, we performed restoration experiments. For this, we cloned full length HA tagged p85 α or p85 β cDNA into *EcoRI/XhoI* site of EGFP expressing MIEG3 retroviral vector. Expression of HA tagged p85 α or p85 β regulatory subunit was restored in MCps by infecting these cells with retroviral supernatants generated from various retroviral vector plasmid. Infected cells were sorted based on EGFP expression, and were used for the experiments.

As seen in Figure 7A, restoring the expression of p85 α in the p85 α -deficient cells completely restored the loss of growth induced by SCF. By comparison, over-expression of p85 β in p85 α -deficient cells did not rescue SCF induced growth. Moreover, over-expression of p85 β in both WT and p85 α -deficient MCps significantly reduced SCF-induced proliferation. These results further demonstrate that p85 α and p85 β subunits play unique roles in both the growth and differentiation of mast cells.

A significant reduction of surface c-Kit expression was also observed in WT and *p85 α* ^{-/-} MCps over expressing p85 β regulatory subunit (Figure 7B). Restoring the expression of p85 α in p85 α -deficient cells completely corrected the defective maturation of these cells. In contrast, over-expression of p85 β in p85 α -deficient MCps did not correct the defective maturation. Furthermore, over-expression of p85 β in WT and p85 α -deficient MCps significantly reduced maturation, as indicated by significantly reduced c-Kit and IgE receptor double-positive cells (Figure 7C), as compared to controls (WT and *p85 α* ^{-/-} MCps transduced with empty vector).

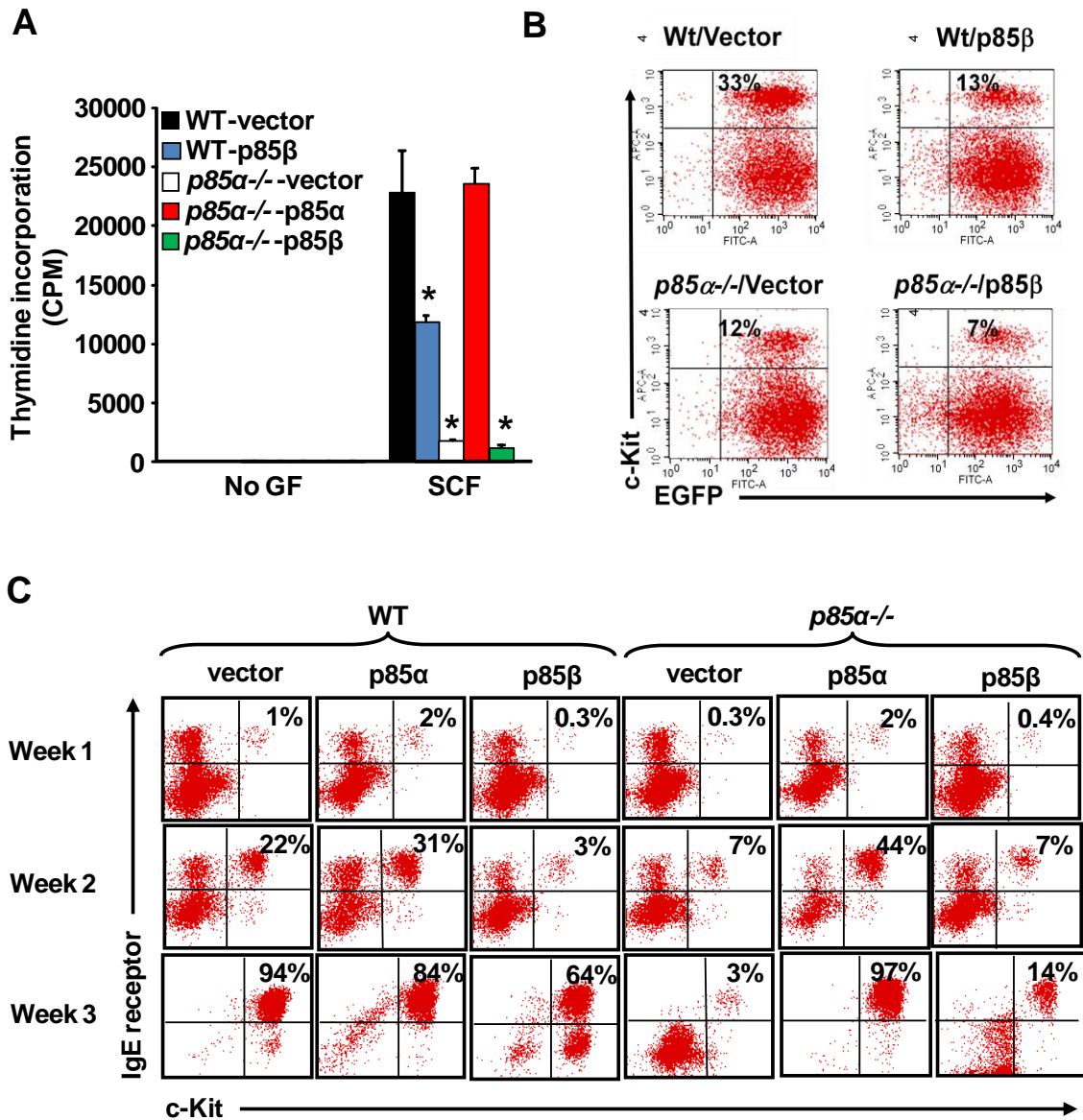


Figure 7. Over-expression of p85β in MCps results in reduced growth and differentiation. WT and *p85α*^{-/-} MCps transduced with vector, p85α or p85β were sorted to homogeneity and cultured in the presence of IL-3 (10 ng/ml). **(A)** Cells were starved for 6 hours in serum- and cytokine-free media and cultured in the presence or absence of SCF (50 ng/ml). After 48 hours, proliferation was evaluated by a [³H] thymidine incorporation assay. Bars represent the mean [³H] thymidine incorporation in BMDCs (CPM ± SD) from one representative experiment performed in quadruplicate. Similar results were observed in three to four additional independent experiments.

* $p < 0.01$, WT-vector vs. WT-p85 β , WT-vector vs. *p85 α* ^{-/-}-vector, WT-vector vs. *p85 α* ^{-/-}-p85 β . Over-expression of p85 β in WT MCps results in reduced c-Kit expression **(B)** and differentiation **(C)**. WT and *p85 α* ^{-/-} MCps transduced with vector, p85 α or p85 β were sorted to homogeneity and cultured in the presence of IL-3 (10 ng/ml). Cells were harvested at indicated time points and maturation was analyzed by staining the cells with antibodies to detect the expression of c-Kit and IgE receptor by flow cytometry. Shown is a dot blot profile of an independent experiment. Similar findings were observed in two to four additional independent experiments. (Results observed in thesis and Figure 7C polished by Raghuveer Mali)

We wished to further examine c-Kit expression and proliferation in response to SCF stimulation in 32D myeloid cell lines. This cell line was co-transfected with c-Kit and p85 α or p85 β to determine the levels of c-Kit expression and proliferation upon over-expression of either of those two subunits. As shown in Figure 8, 32D cells that over-express p85 β showed reduced c-Kit expression and lowered proliferation in response to SCF stimulation, result that is consistent to that seen in experiments with MCps.

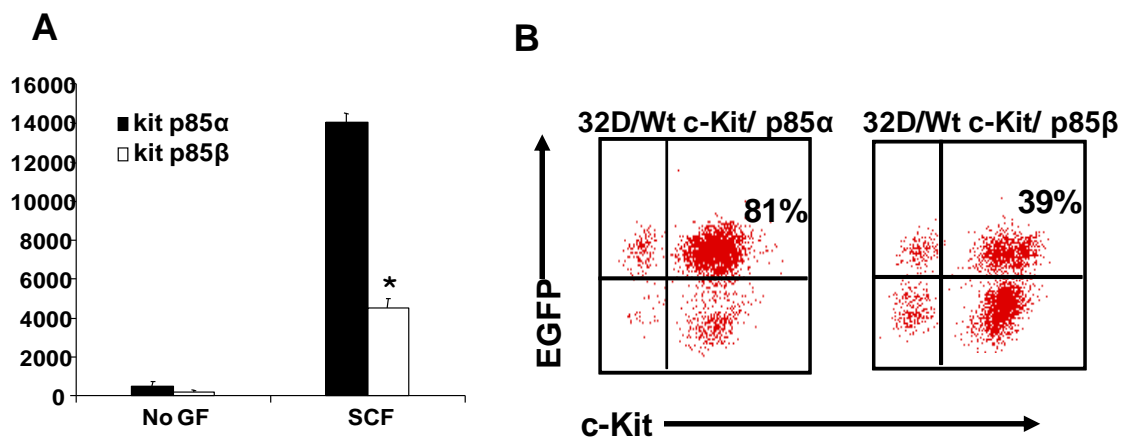


Figure 8. The p85 β regulatory subunit of PI-3K binds to c-Kit and is activated upon SCF stimulation. 32D cells were co-infected with c-Kit and p85 α or p85 β regulatory subunits. **(A)** After sorting for c-Kit and EGFP double-positive cells, these cells were subjected to a proliferation assay to evaluate how these cells responded to SCF stimulation. 32D cells transduced with c-Kit and p85 β proliferate significantly less than p85 α -transduced 32D cells. **(B)** Over-expression of p85 β results in reduced surface c-Kit expression in 32D cells.

IV. Differential roles of p85 α and p85 β regulatory subunits in mast cell biology

Given the fact that p85 β cannot compensate for the loss of p85 α in mast cell biological functions, we then evaluated differential roles of p85 α and p85 β regulatory subunits in mast cell growth, survival and maturation.

A. p85 α and p85 β differentially regulate mast cell growth in response to SCF stimulation

To compare the roles of p85 α and p85 β in mast cell growth, cell proliferation was assessed in mast cell progenitors (MCps) from WT, *p85 α ^{-/-}* and *p85 β ^{-/-}* mice. MCps from WT and knockout mice were starved for serum and cytokines for 6 h, after which they were cultured in the presence or absence of SCF for 48 h. Cells were then pulsed with [³H] thymidine for 6 h. Thymidine incorporation was measured, which is a direct indicator of these cells' proliferation rate in response to SCF stimulation. MCps from WT, *p85 α ^{-/-}* and *p85 β ^{-/-}* mice grown in the absence of growth factors showed minimal thymidine incorporation (Figure 9). In contrast to loss of p85 α regulatory subunits, which significantly reduced mast cell proliferation in response to SCF stimulation (Figure 9A), loss of p85 β significantly enhanced mast cell growth compared to WT controls in response to SCF (Figure 9B). These results suggest that p85 α and p85 β regulatory subunits of PI-3K differentially regulate mast cell growth in response to SCF stimulation.

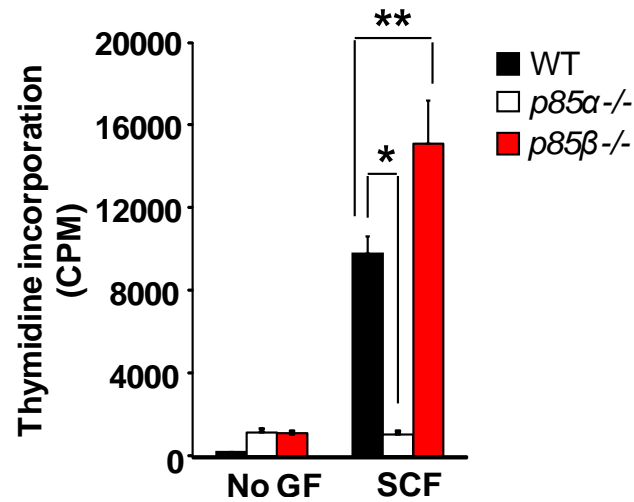


Figure 9. *p85α* and *p85β* regulatory subunits differentially regulate mast cell growth in response to SCF stimulation. MCps from WT, *p85α*^{-/-} and *p85β*^{-/-} mice were starved for 6 hours in serum- and cytokine-free media and cultured in the presence or absence of SCF (50 ng/ml). After 48 hours, proliferation was evaluated by [³H] thymidine incorporation. Bars represent the mean [³H] thymidine incorporation in MCps (CPM \pm SD) from one representative experiment performed in quadruplicate. Similar results were observed in six independent experiments. **p*<0.01, WT vs. *p85α*^{-/-}. ***p*<0.01, WT vs. *p85β*^{-/-}.

B. p85 α and p85 β differentially regulate mast cell survival in response to SCF stimulation

We next evaluated the roles of p85 α and p85 β regulatory subunits in mediating mast cell survival in response to SCF stimulation. *In vitro* cultures of low-density bone marrow cells harvested from WT, p85 $\alpha^{-/-}$ and p85 $\beta^{-/-}$ mice were starved for 6 h and then grown in the presence or absence of SCF. After 48 h, apoptosis was assessed by staining the cells with antibodies to Annexin V and 7-AAD followed by flow cytometry analysis. As seen in Figure 10, only 1–2% of MCps from WT, p85 $\alpha^{-/-}$ and p85 $\beta^{-/-}$ mice survived in the absence of any growth factors for 48 h as determined by the presence of Annexin V– and 7-AAD–negative cells. Although WT MCps showed increased survival in the presence of SCF (60%), the deficiency of p85 α significantly reduced the survival of MCps (6%). In contrast, p85 β -deficient MCps demonstrated significantly enhanced survival in the response to SCF stimulation (69%) compared to WT controls (Figure 10). The results suggest differential roles of p85 α and p85 β in mast cell survival.

We then examined the cell cycle progression in WT, p85 $\alpha^{-/-}$ and p85 $\beta^{-/-}$ MCps in response to SCF stimulation. Starved and SCF stimulated (50 ng/ml) MCps were labeled with propidium iodide and cell cycling was analyzed by flow cytometry. In contrast to p85 α deficiency, lack of p85 β regulatory subunit did not affect the cell cycle, as shown by significant progression of p85 β -deficient MCps to S phase in response to SCF stimulation (Figure 11).

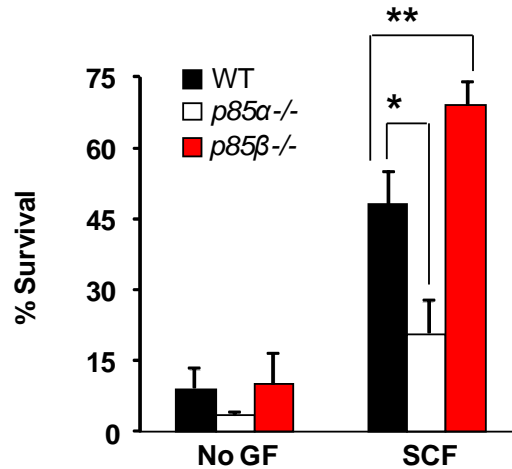


Figure 10. *p85α* and *p85β* regulatory subunits differentially regulate mast cell survival. MCps from WT, *p85α*^{-/-} and *p85β*^{-/-} mice were starved for 6 hours in serum- and cytokine-free media and cultured in the presence or absence of SCF (50 ng/ml). After 48 hours, cells were stained with phycoerythrin-conjugated annexin V and 7-AAD followed by flow cytometry analysis. Shown is a representative bar graph demonstrating percentage of annexin V and 7-AAD negative cells in the presence and absence of SCF. Similar results were observed in four independent experiments. **p* < 0.05, WT vs. *p85α*^{-/-}. ***p* < 0.01, WT vs. *p85β*^{-/-}.

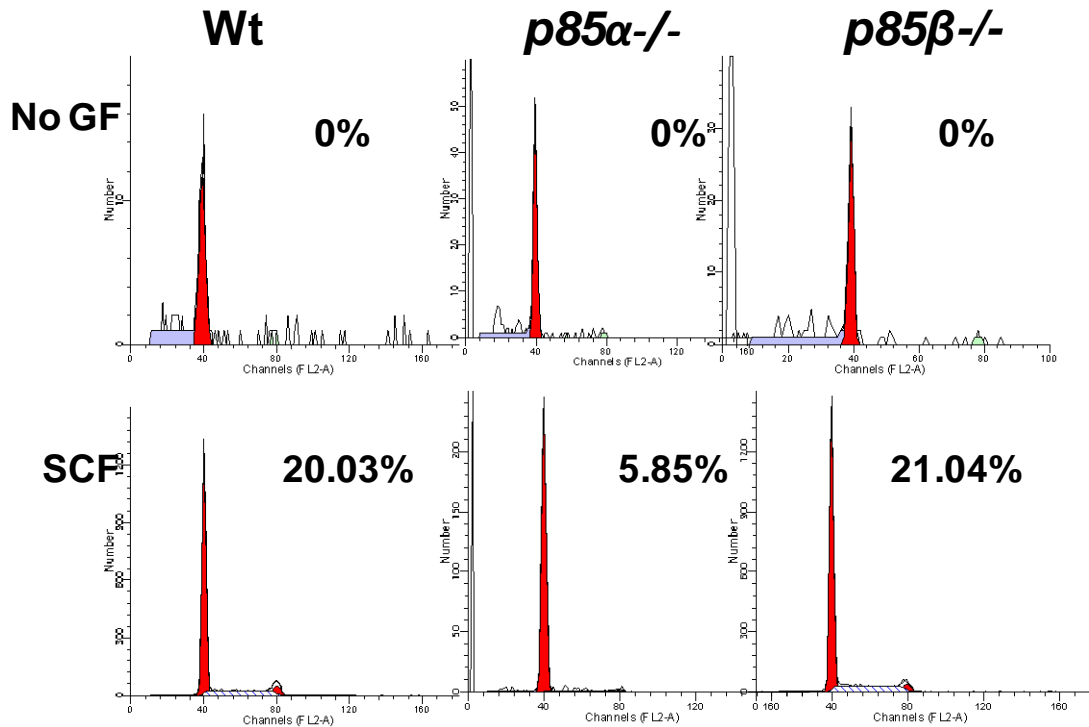


Figure 11. *p85α* and *p85β* regulatory subunits differentially regulate the cell cycle of mast cells'. WT, *p85α*^{-/-} and *p85β*^{-/-} MCps were starved for 6 h and equal numbers of cells were plated into individual well of a 24-well plate. Cells were either cultured in cytokine-free culture medium (control) or in medium supplemented with SCF (100 ng/ml) for 48 h. Cells were stained with propidium iodide, and then analyzed by flow cytometry. The percentage of cells in S phase of the cell cycle is shown.

C. p85 α and p85 β differentially regulate maturation of MCps

While our aforementioned studies clearly demonstrate a functional dichotomy in cell cycle progression between the two regulatory subunits in response to SCF, we wanted to assess how the loss of p85 α and p85 β alters maturation in response to IL-3. Low-density bone marrow cells from WT, p85 $\alpha^{-/-}$ and p85 $\beta^{-/-}$ mice cells were harvested and cultured in media supplemented with IL-3 (10 ng/ml). Maturation into mast cells was evaluated based on expression of two mast cell surface markers: c-Kit and the IgE receptor. Mast cell maturation was analyzed on a weekly basis by staining MCps with antibodies to detect the expression of c-Kit and IgE receptors by flow cytometry. Figure 12 represents a dot blot profile for the maturation of these mast cells. While 91% of WT MCps fully matured into c-Kit and IgE receptor double-positive cells, after four weeks, only 20% of all p85 α -deficient MCps had matured. By contrast, a p85 β deficiency did not impair maturation to mast cells, with 97% maturation to mast cells by the end of 4 weeks. These results demonstrate that p85 α and p85 β differentially regulate the maturation of mast cells.

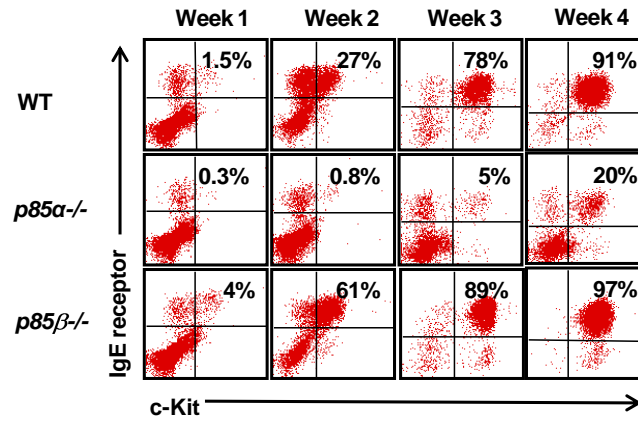


Figure 12. *p85α* and *p85β* differentially regulate mast cell maturation. LDMNCs from WT, *p85α*^{-/-} and *p85β*^{-/-} mice were cultured in the presence of IL-3 (10 ng/ml) for 4 weeks. At indicated time points, maturation was analyzed by staining the cells with antibodies that recognize c-Kit and IgE receptor followed by flow cytometry. Shown is dot blot profile from one of four independent experiments.

D. The p85 β regulatory subunit of PI-3K binds to c-Kit and becomes activated upon SCF stimulation

To assess the molecular basis for differential roles of p58 α or p85 β regulatory subunits, we first examined whether the regulatory subunits bind to c-Kit upon stimulation with ligand SCF. The 32D cells that expressed c-Kit and p58 α or p85 β , were deprived of serum and growth factors for 12 h. These cells were stimulated with 100 ng/ml of recombinant rat SCF for 5 min, followed by lysis and immunoprecipitation using an anti-c-Kit antibody. The immunoprecipitated proteins were then subjected to Western blot analysis using anti-HA antibody. As seen in Figure 13, when c-Kit is activated by its ligand SCF both p85 α and p85 β binds equally to c-Kit. We next examined whether these HA-tagged regulatory subunits are activated to the same extent upon binding to c-Kit. To address this question, we performed immunoprecipitations using the same lysates and anti-HA antibody, and the immunocomplexes were subjected to Western blot analyses using anti phospho-tyrosine antibody. As shown in Figure 13, both p85 α and p85 β that were bound equally to c-Kit were activated upon SCF stimulation.

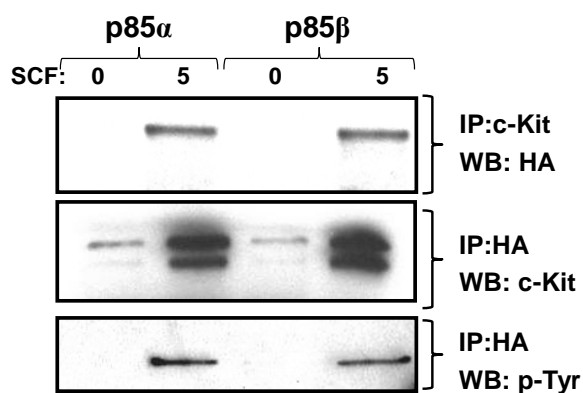


Figure 13. The p85 β regulatory subunit of PI-3K binds to c-Kit and is activated upon SCF stimulation. 32D cells expressing WT c-Kit and p85 α or p85 β were starved for 8 hours and stimulated with SCF (100 ng/ml) for 5 min. Equal amount of cell lysates (500 μ g) were subjected to immunoprecipitation with an anti-c-Kit antibody followed by western blot analysis with an anti-HA antibody or immunoprecipitated with an anti-HA antibody followed by western blot analysis with an anti-c-Kit antibody or anti-phosphotyrosine antibody.

E. p85 α and p85 β differentially regulate c-Kit receptor-mediated signaling events in MCps

The signaling molecules AKT, ERK and JNK MAP kinase play critical roles in c-Kit-mediated proliferation and survival of mast cells (Rottapel, Reedijk et al. 1991; Duronio, Welham et al. 1992; Chian, Young et al. 2001). In an effort to determine the mechanism behind differential regulation of mast cell growth and survival by p85 α and p85 β , we measured the phosphorylation of these regulatory subunits in WT, p85 $\alpha^{-/-}$ and p85 $\beta^{-/-}$ MCps in response to SCF. As seen in Figure 14, p85 α -deficient MCps showed significantly reduced activation of AKT, ERK and JNK MAP kinase in response to SCF compared to WT controls. In contrast, enhanced activation of ERK and JNK MAP kinase—but not AKT—was observed in p85 β -deficient MCps compared to WT controls in response to SCF stimulation. These results suggest that differential regulation of mast cell growth and survival by p85 α and p85 β subunits are due to differential activation of ERK and JNK MAP kinase downstream from c-Kit.

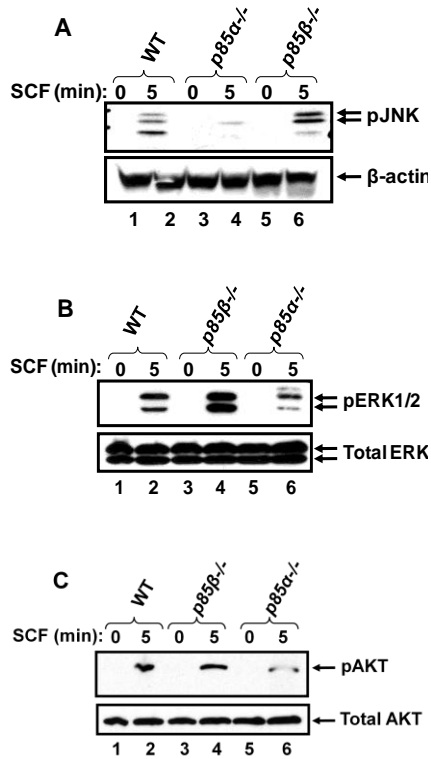


Figure 14. Reduced activation of AKT, ERK and JNK in *p85α*^{-/-} MCps in response to SCF stimulation. WT, *p85α*^{-/-} and *p85β*^{-/-} MCps were starved overnight in serum- and cytokine-free media and stimulated with SCF (100 ng/ml) for 5 min. Equal amount of protein lysates were subjected to western blot analysis using an anti-phospho-JNK, anti-phospho-AKT or anti-phospho-ERK1/2 antibody. The levels of β-actin, and total AKT and ERK proteins were used as loading controls (**A-C**). Similar findings were observed in two independent experiments.

V. p85 β negatively regulates c-Kit receptor signaling by binding to phosphorylated E3 ubiquitin ligase, c-Cbl

A. p85 β negatively regulates c-Kit receptor internalization and degradation

Activation of c-Kit receptor by its ligand SCF is followed by its internalization and degradation (Levkowitz, Waterman et al. 1998; Lee, Wang et al. 1999; Miyake, Mullane-Robinson et al. 1999; Taher, Tjin et al. 2002). To determine the mechanism behind increased activation of signaling events downstream from the c-Kit receptor in p85 β -deficient cells, we examined the rate of internalization of the c-Kit receptor in response to SCF in p85 β -deficient MCps and also in murine myeloid 32D cells co-infected with c-Kit and p85 α or p85 β . As seen in Figure 15, a deficiency of p85 β in MCps inhibited the rate of SCF-induced c-Kit receptor internalization compared to WT controls at all time points examined.

We next performed studies to evaluate the levels of c-Kit receptor degradation in 32D cells co-expressing c-Kit and p85 α or p85 β in response to SCF. After SCF stimulation, there was significantly enhanced c-Kit receptor internalization and degradation in p85 β over-expressing cells compared to p85 α over-expressing cells (Figures 16 and 17A). These results were observed despite similar binding affinities of p85 α and p85 β to the c-Kit receptor in response to SCF stimulation. Taken together, these results suggest that p85 β negatively regulates c-Kit receptor signaling in part by regulating its rate of internalization and degradation.

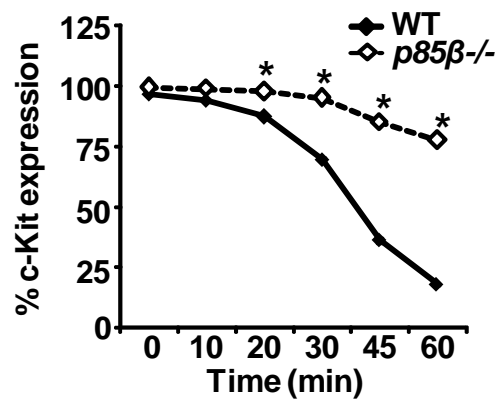


Figure 15. Reduced c-Kit receptor internalization in *p85β*-deficient MCps compared to WT in response to SCF. WT or *p85β*^{-/-} MCps were starved overnight and incubated with cyclohexamide (100 ng/ml) for 2 hours. After cyclohexamide treatment, cells were stimulated with SCF (100 ng/ml) for indicated time points and c-Kit receptor internalization was studied by staining the cells with PE-conjugated anti-c-Kit receptor antibody followed by flow cytometric analysis. Shown is one of four independent experiments performed in triplicate. **p*<0.01, WT vs. *p85β*^{-/-}.

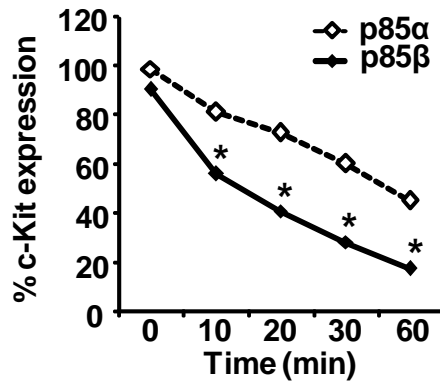


Figure 16. Enhanced. c-Kit receptor internalization in 32D cells over-expressing p85β as compared to p85α subunit upon SCF stimulation. 32D cells co-infected with c-Kit and p85α or p85β were starved for 8 hours and incubated with cyclohexamide (100 ng/ml) for 2 hours. After cyclohexamide treatment, cells were stimulated with SCF (100 ng/ml) for indicated time points and c-Kit receptor internalization was studied by staining the cells with PE-conjugated anti-c-Kit receptor antibody followed by flow cytometric analysis. Shown is one of six independent experiments performed in triplicate. *p<0.01, p85α vs. p85β.

B. Cells over-expressing p85 β demonstrate enhanced activation of E3 ubiquitin ligase c-Cbl, compared to cells over-expressing p85 α

Previous studies have shown that c-Kit internalization and degradation is regulated largely by an E3 ubiquitin ligase c-Cbl (Levkowitz, Waterman et al. 1998; Lee, Wang et al. 1999; Miyake, Mullane-Robinson et al. 1999; Taher, Tjin et al. 2002). We hypothesized that p85 α and p85 β may differentially regulate the activation and binding of c-Cbl in response to c-Kit activation. To test this, we first studied the activation of c-Cbl in 32D cells over-expressing p85 α or p85 β . Significantly enhanced phosphorylation of c-Cbl was found in 32D cells over-expressing p85 β as compared to 32D cells over-expressing p85 α (Figure 19B).

C. p85 β regulatory subunit of PI-3K shows enhanced binding to E3 ubiquitin ligase, c-Cbl, as compared to p85 α regulatory subunit of PI-3K

We next performed immunoprecipitation experiments in 32D cells expressing an HA-tagged version of p85 α or p85 β . These transfected cells were stimulated with SCF, followed by Western blot analysis using an anti-phospho-c-Cbl antibody. Significantly enhanced binding of phospho-c-Cbl to p85 β was observed compared to p85 α in response to SCF stimulation (Figure 17C).

D. 32D cells over-expressing p85 β show enhanced c-Kit ubiquitination upon SCF stimulation as compared to those over-expressing p85 α

We then examined ubiquitination of the c-Kit receptor in 32D cells over-expressing p85 α or p85 β . The 32D cells were starved of serum and growth factors, and then were treated with cycloheximide, followed by SCF for upto 30 minutes at 37°C. Cell lysates were incubated with anti-c-Kit antibody, immunoprecipitated and separated by SDS-PAGE, and then analyzed by western blotting using anti-ubiquitin antibody. Enhanced

ubiquitination of c-Kit bands was detected in response to SCF stimulation in 32D cells over-expressing p85 β . Interestingly, there was enhanced binding of c-Cbl to p85 β coincident with the increased ubiquitination of c-Kit in cells over-expressing p85 β . This complex between c-Cbl to p85 β was much reduced in cells over-expressing p85 α . Taken together, these results suggest that p85 β negatively regulates c-Kit receptor signaling in part by regulating its ubiquitination and degradation.

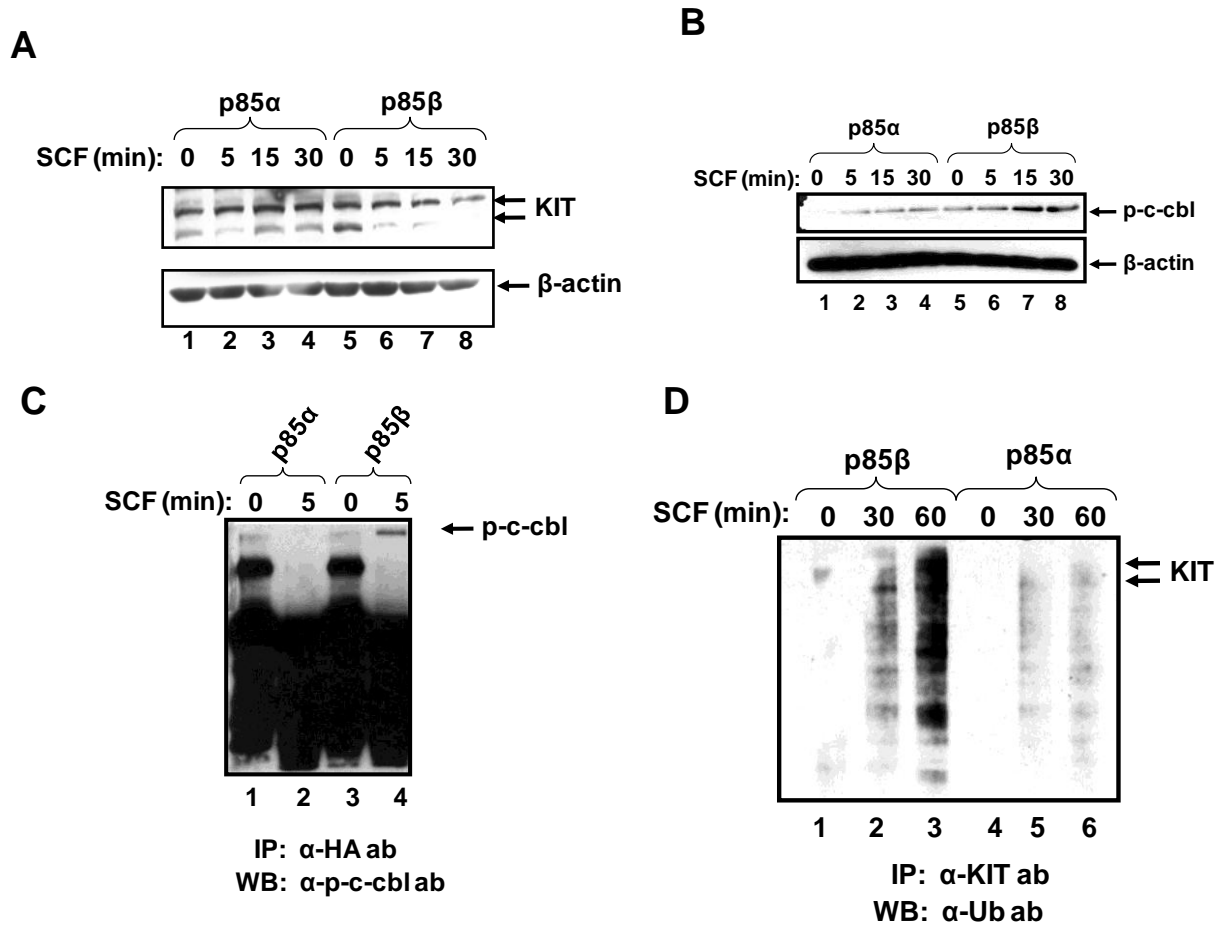


Figure 17. Enhanced c-Kit degradation and c-Cbl activation in 32D cells over-expressing p85β regulatory subunit upon SCF stimulation. 32D cells co-infected with c-Kit and p85α or p85β were starved and incubated with cyclohexamide (100 ng/ml), followed by stimulation with SCF. c-Kit expression in response to SCF stimulation was evaluated by performing Western blot analysis using anti-c-Kit antibody **(A)** and anti-phospho-c-Cbl antibody **(B)**. **(C)** 32D cells co-infected with c-Kit and p85α or p85β were starved for 8 h and stimulated with SCF (100 ng/ml) for 5 min. Equal amounts of cell lysates (500 μg) were immunoprecipitated with an anti-HA antibody, followed by Western blotting with a phospho-c-Cbl antibody. p85β preferentially binds to phospho c-Cbl compared to p85α in response to SCF stimulation. **(D)** 32D cells co-infected with c-

Kit and p85 α or p85 β regulatory subunits were starved and incubated with cyclohexamide, followed by stimulation with SCF. To evaluate c-Kit ubiquitination in response to SCF treatment, equal amounts of cell lysate were subjected to immunoprecipitation using c-Kit antibody, followed by Western blotting using an anti-ubiquitin antibody. Shown is representative data from one of three independent experiments.

Our study implies functional uniqueness for PI-3K regulatory subunits in mast cell development. p85 α deficiency impairs growth, maturation and survival of mast cells; whereas p85 β deficiency enhances c-Kit signaling in part by inhibiting c-Kit internalization, ubiquitination and degradation. Although p85 α and p85 β share 100% protein domain homology, amino acid comparison shows wide disparity in amino acid composition of SH3 and BH domains (Figure 18). Interestingly, p85 α ^{-/-} MCps show severe phenotypic defects, irrespective of the presence of shorter regulatory subunits p50 α and p55 α . p50 α and p55 α are splice variants of p85 α , but lack amino terminal domains of SH3 and BH. Based on these facts, we hypothesized that the functional uniqueness of p85 α regulatory subunit might be in part imparted by its amino terminal domains (SH3 and BH).

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p85alpha  -M AEGYQYRALYDYKKEREEDIDLHGLDILTVNKGSLVALGFS DGQEARFEDIGWNGY 59
p85beta   MA AEGFYQYRAVYPPRRRERPELELLPGDLLVVSRLVALQALGVADGGERCENHVGWMPGF 60
          *****
p85alpha  NETTGERGDFPGTYVEYIGRKRI PETEKPRPPRPPLVAPSSSKTEADTEQQALPLPDLLA 119
p85beta   NERTRQRGDFPGTYVEFLGTFVAL REGPRPRGPRPLPARPLDGSSES-----GHILPDLLA 115
          *****
p85alpha  EQFAPPDVAPPLLKILEAEKKKGLCSTLYRTQSSSNPAELRQLLDCDAASVDLEHIDV 179
p85beta   EQESPDPAPPILVKIVEAEQAELESECYSKPELPAT-----RTDWSLSLEQWDR 167
          *****
p85alpha  HVLADAFKRYLADLPNPVIVAVYNEMMSLAQELQSPEDCIQLLKKLIRLPNIPHCWLT 239
p85beta   TALYDAVKGFLALAAAVTPEAAAEAYRALREVAGE-----VGLVLEPPTLELHQALT 221
          *****
p85alpha  LQYLLKHFFKLSQASSKNLLNARVISEIESVLFKFFPAASSDN-----TEHLIKAEIL 293
p85beta   LRFLQLHLGRVARRAPSPDTAVHAIASAFGELLRIIPSGGEGDGSEFVPDFPVLLERL 281
          *****
p85alpha  ISTEWNERRPAD-ALPPKPP--ETTVAANSMNNMNSLQDAENYIGDISREEVNERLRDT 350
p85beta   VQEHVEEQAARFALPPKPKKAPAPTALANGGSPPSLQDAENYIGDISREEVNERLRDT 341
          *****
p85alpha  ADGTFELVRDASTKMHGDYTLTPRKGGNNKLIKIFHRDGKYGFSDDLTFNSVVELINHYRN 410
p85beta   PDGTFELVRDASSKIQGEYTLTLRKGGNNKLIKVFHRDGHYGFSEPLTECSVVELISHYRH 401
          *****
p85alpha  ESLAQYNPKLDVLLYPVSKYQQDQVVKEDNIEAVGKKLHEYNTQFQKKSREYDRLYEY 470
p85beta   ESLAQYNAKLDTRLLYPVSKYQQDQVVKEDSVEAVGAQLKVYHQYQDKSREYDQLYEY 461
          *****
p85alpha  TRTSQEIQMKRTAIEAFNETIKIFEEQCQTQERYSKYEIEKFKREGNEKEIQRIMHNDK 530
p85beta   TRTSQELQMKRTAIEAFNETIKIFEEQGQTQEKCSKEYLERFERREGNEKEMQRILLNSER 521
          *****
p85alpha  LKSRISSEIIDRRRLLEEDLKKQAAEYREIDKRMNSIKPDLIQLRKT RDQYLMWLTQKGV 590
p85beta   LKSRIAEIHESRTKLEQDLRAQASDNREIDKRMNSLKPDLMQLRKIRDQYLVWLTQKGAR 581
          *****
p85alpha  QKKLNEWLGNE-NTEDQYSLVEDDEDLPHHDEKTNVGSNRRNKAENLLRGKRDGTFLVR 649
p85beta   QRKINENLGIKNETEDQYSLMEDEDALPHHEERTWYVGKINRTQAEEMLSGKRDGTFLIR 641
          *****
p85alpha  ESSKQGCYACSVVVDGEVKKCVINKTATGYGFAEPYNLYSLKELVLVHYQHTSLVQHND 709
p85beta   ESSQRCYACSVVVDGDTKHCVIYRTATGFGFAEPYNLYGSLKELVLVHYQHASLVQHND 701
          *****
p85alpha  LNYTLAYPVY AQRR----- 724
p85beta   LTYTLAHEVR APGPGPPSAAR 722
          *****

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Figure 18. Sequence comparison between p85 α and p85 β regulatory subunits of PI-3K. The sequences of the p85 α and p85 β regulatory subunits were compared using the UniProt Multiple Sequence Alignment tool. Shaded areas represent identical amino acid residues shared between the p85 α and p85 β . p85 α and p85 β regulatory subunits share domain homology from the proteins' amino to carboxy terminus. The corresponding domain positions are enclosed in boxes: SH3 domain in blue, proline-rich regions in red, BH domain in green, amino-SH2 in purple, and carboxy-SH2 in yellow. As represented, the two regulatory subunits share lesser identity of amino acids at their amino terminal domains of SH3 and BH. The carboxy terminal portion of the regulatory subunits, which includes the amino-SH2, inter-SH2, and carboxy-SH2 regions, shares more sequence identity.

VI. Cooperation between the SH3 and BH domains of p85 α is required for mast cell growth, but not maturation

To evaluate importance of SH3 and BH domains of p85 α in mast cell functions, we used two approaches (i) First, the *collective* contributions of the SH3 and BH domains of p85 α to mast cell biology were addressed by reconstituting and over-expressing p50 α in *p85 α ^{-/-}* MCps. (ii) The second approach to analyze the *individual* contributions of the SH3 and BH domains of p85 α to mast cell functions involved expressing of different p85 α mutants in *p85 α ^{-/-}* MCps that lack either SH3 or BH domain.

A. Over-expression of the p50 α regulatory subunit in *p85 α ^{-/-}* MCps corrects maturation; and partially corrects growth in response to SCF stimulation

To assess the extent to which the shorter isoforms of p50 α contribute to mast cell maturation and proliferation, we cloned an HA-tagged p50 α gene into a MIEG3 bicistronic retroviral vector that expresses EGFP via an internal ribosomal entry site. Viral supernatants were generated and were used to infect WT and *p85 α ^{-/-}* MCps. Expression of HA-tagged p85 α and p50 α constructs were confirmed in EGFP-sorted *p85 α ^{-/-}* cells (Figure 19A). As expected, restoration of full-length p85 α completely corrected the observed defect in mast cell maturation associated with p85 α -deficient cells (Figure 19B). Over-expression of p50 α in p85 α -deficient cells also completely corrected the defective mast cell maturation to WT levels in *p85 α ^{-/-}* MCps. As seen in Figure 19C the expression of full length p85 α in *p85 α ^{-/-}* MCps restores the SCF-induced growth to WT levels. In contrast, there was only 50% rescue in SCF-induced proliferation in p85 α -deficient cells over-expressing the p50 α subunit. These results suggest that the shorter p50 α do contributes to mast cell differentiation and proliferation. However, the full-length form of p85 α is indispensable for mast cell functions. These results suggest

that the amino terminal domains of SH3 and BH collectively play an important role in mediating SCF-induced growth in MCps.

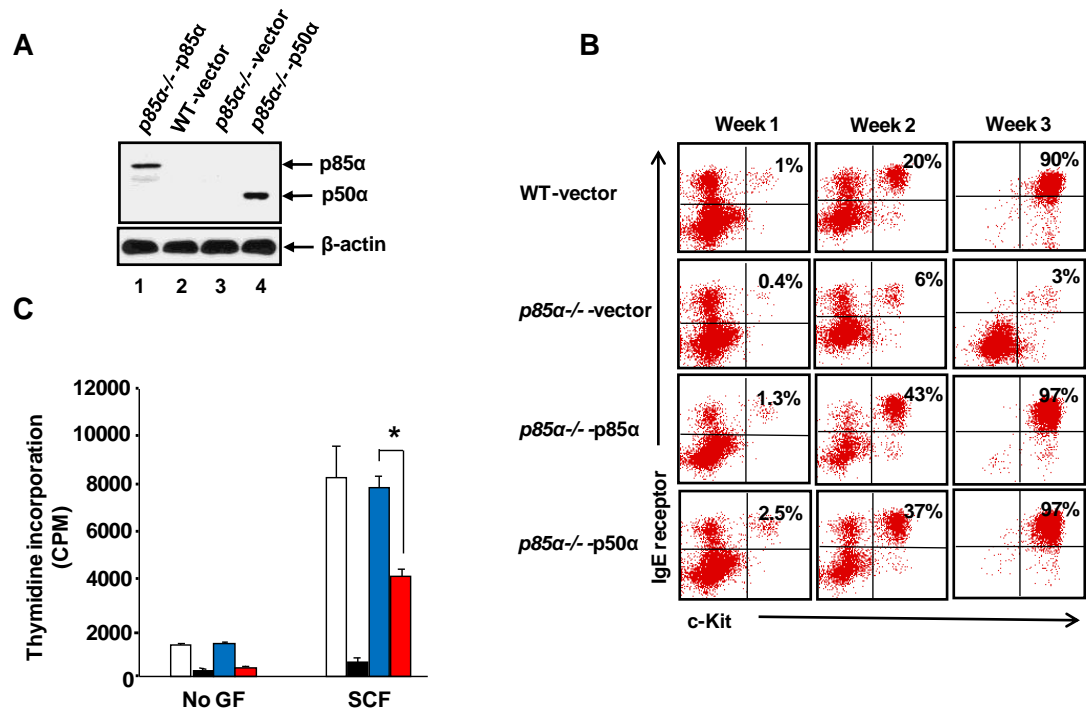


Figure 19. Over-expression of the PI-3kinase regulatory subunit of p50α restores maturation and partially restores growth in response to SCF in *p85α*^{-/-} MCps. (A) Expression of p50α in *p85α*^{-/-} MCps. MCps from WT and *p85α*^{-/-} mice were transduced with vector, HA tagged full length p85α or p50α and sorted to homogeneity. Expression of p85α and p50α in sorted cells was analyzed by Western blotting using anti-HA and β-actin antibodies. **(B)** Restoration of p50α corrects the defective maturation of *p85α*^{-/-} deficient BMMCs. BMMCs in (d) were collected at indicated time points. Mast cell differentiation was analyzed by staining the cells with antibodies that recognize c-Kit and IgE receptor followed by flow cytometric analysis. Shown is a representative dot blot profile from five independent experiments. *p < 0.05, WT vs. *p85α*^{-/-}-vector. **(C)** Over-expression of p50α partially correct the defective growth of *p85α*^{-/-} MCps. WT and

p85α^{-/-} MCps expressing the indicated constructs were starved of growth factors and stimulated with SCF. After 48 hours, proliferation was evaluated by [³H] thymidine incorporation. Bars represent the mean [³H] thymidine incorporation (CPM ± SD). Shown is data from one of many independent experiments. **p*<0.05, *p85α^{-/-}* -*p85α* vs. *p85α^{-/-}* -*p50α*. (Result observed in this thesis and Figure 19B polished by Raghuveer Mali)

We then evaluated the importance of the individual contributions of p85α's SH3 and BH domains to mast cell function.

B. Amino terminal mutants of p85α (p85αΔSH3 and p85αΔBH) rescue maturation and Mitf expression in p85α^{-/-} MCps

HA-tagged cDNA encoding p85α, p85αΔSH3 or p85αΔBH were cloned into a MIEG3 retrovirus (Figure 20A) and *p85α^{-/-}* bone marrow cells were transduced with viral supernatants expressing the indicated variants of the p85α regulatory subunit. These cells were sorted to homogeneity based on EGFP expression. Expression of various HA-tagged constructs in MCps were confirmed by Western blot analysis using an anti-HA antibody (Figure 20B), and were used for studies described here. As seen in Figure 20C, expression of p85α mutants p85αΔSH3 and p85αΔBH in the p85α-deficient cells completely corrected the defect in mast cell maturation, as indicated by restoration of c-Kit and IgE receptor double positive population. These results further confirm that the amino terminal domains of p85α are not essential for mast cell maturation.

Maturation of mast cells is driven by a complex network of transcription and growth factors. Microphthalmia-associated transcription factor (Mitf) is known to be a key regulator of c-Kit expression and maturation of mast cells (Kasugai, Oguri et al. 1993; Isozaki, Tsujimura et al. 1994; Kitamura, Morii et al. 2002). To determine how p85α and

p85 α mutants p85 α Δ SH3 or p85 α Δ BH restore mast cell maturation in p85 $\alpha^{-/-}$ MCps, we analyzed the expression of Mitf in p85 α -deficient cells by transducing them with the full-length form of p85 α or one of the mutants p85 α Δ SH3 or p85 α Δ BH. As seen in Figure 20D, the p85 α -deficient cells showed significant reduction in expression of Mitf compared to WT controls cells. Expression of p85 α or one of the p85 α mutants (either p85 α Δ SH3 or p85 α Δ BH) in p85 α -deficient cells restored Mitf expression. These results suggest that p85 α regulates the expression of Mitf, which, in turn, controls mast cell maturation.

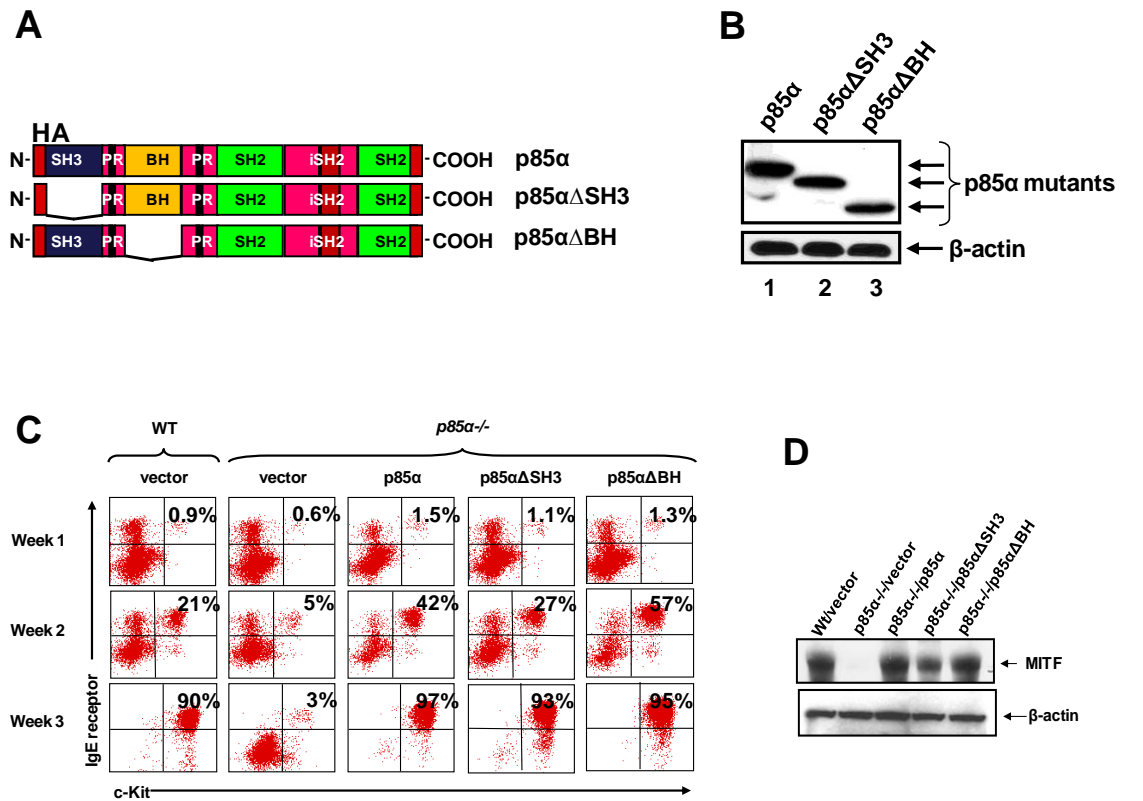


Figure 20. Amino terminal mutants of p85α (p85αΔSH3 and p85αΔBH) rescue maturation and Mitf expression in p85α^{-/-} MCps. (A) Schematic of full length p85α and p85α mutants lacking either the SH3 or the BH domain. Full length p85α or p85α mutants lacking either the amino terminal SH3 domain (1-80 a.a) or the BH domain (102-288 a.a) were cloned into a bi-cistronic retroviral vector MIEG3. The constructs were HA tagged at the amino terminus to distinguish exogenous expression from endogenous p85 protein. (B) Expression of full length p85α or p85α mutants. MCps from p85α^{-/-} mice were transduced with vector, full length p85α or p85α mutants lacking either the SH3 domain (p85αΔSH3) or the BH domain (p85αΔBH) and sorted to homogeneity. Cells were harvested and subjected to Western blot analysis using an anti-HA antibody or β-actin antibody as indicated. Expression of various p85α mutants is indicated in the upper panel. (C) Expression of p85α mutants into p85α^{-/-} MCps corrects

defective mast cell differentiation. At indicated time points, maturation was evaluated by staining the cells with antibodies that recognize c-Kit and IgE receptor by flow cytometry. Shown is a representative dot blot **(C)** from five independent experiments. * $p < 0.05$, WT vs. $p85\alpha^{-/-}$ -vector. **(D)** Sorted cells grown in the presence of IL-3 (10 ng/ml) were harvested and subjected to Western blot analysis using an anti-Mitf antibody and a β -actin antibody, as indicated. (Result observed in thesis and Figure 20C polished by Baskar Ramdas)

C. The amino terminal domains of $p85\alpha$ ($p85\alpha\Delta SH3$ and $p85\alpha\Delta BH$) are critical for growth of $p85\alpha^{-/-}$ MCps

Because the growth defects in $p85\alpha^{-/-}$ MCps could not completely restored in spite of the over expression of $p50\alpha$ which lacks SH3 and BH domains of $p85\alpha$, we hypothesized that the amino terminal SH3 and BH domains of $p85\alpha$ must contribute significantly to the growth of MCps in response to SCF stimulation. To test this idea, we performed proliferation assays on sorted $p85\alpha^{-/-}$ MCps transduced with full-length and mutant versions of $p85\alpha$ which lacked either the SH3 or BH domains. As seen in Figure 21A, the $p85\alpha^{-/-}$ MCps transduced with only the empty vector showed significantly reduced mast cell growth in response to SCF stimulation. This indicates that the $p85\alpha$ regulatory subunit is important for mediating the signals downstream of c-Kit to induce mast cell growth in response to SCF stimulation. Reconstitution of $p85\alpha^{-/-}$ with full-length $p85\alpha$ cDNA rescued the growth defect to WT levels. Interestingly, the reconstitution of mutant constructs ($p85\alpha\Delta SH3$ or $p85\alpha\Delta BH$) could only marginally rescue the mast cell growth, despite the fact that truncated regulatory subunits completely corrected the maturation defect and c-Kit levels in $p85\alpha^{-/-}$ MCps (Figure 20C). This demonstrates that the amino terminal domains (SH3 and BH) work cooperatively to affect mast cell growth in response to SCF stimulation, and signals mediated via the SH3 and BH domains of

p85 α are critical for inducing c-Kit mediated growth in mast cells in response to SCF stimulation.

D. The amino terminal domains of p85 α (p85 α Δ SH3 and p85 α Δ BH) are critical for survival of p85 α ^{-/-} MCps

Although mutant p85 α constructs (p85 α Δ SH3 and p85 α Δ BH) restored maturation of p85 α ^{-/-} MCps to WT levels (Figure 20C), SCF-induced growth could only be partially restored in these cells (Figure 21A). We wished to determine whether the reduced proliferation in the absence of p85 α SH3 or BH domains is caused by reduced survival potential of these cells in response to SCF stimulation. We transduced p85 α ^{-/-} MCps with empty vector, full-length p85 α , p85 α Δ SH3 or p85 α Δ BH, and then starved these cells, followed by treatment with SCF for 48 h. Apoptosis was assessed using Annexin V and 7-AAD staining. As seen in Figure 21B, MCps lacking p85 α failed to survive in response to SCF stimulation. Expression of full-length p85 α completely corrected the survival defect in response to SCF stimulation of the transduced p85 α ^{-/-} MCps. This indicates that p85 α plays a critical role in mast cell survival in response to SCF. By contrast, p85 α lacking either the SH3 (p85 α Δ SH3) or BH (p85 α Δ BH) domains showed reduced survival. This result suggests that the amino terminal SH3 and BH domains of the p85 α regulatory subunit are critical for the survival of MCps in response to SCF stimulation, possibly by mediating some downstream signals.

E. The amino terminal SH3 and BH domains of p85 α are important for SCF-induced AKT, ERK, and JNK1 activation, but not JNK2 activation

We then examined the activation of downstream c-Kit receptor signaling in response to SCF stimulation when SH3 or BH domains were absent. As seen in Figure 21C, activation of AKT, ERK, and JNK MAP kinase was restored to WT levels in p85 α ^{-/-}

MCps expressing the full-length p85 α ; whereas only a partial correction in the activity of these molecules was observed in *p85 α* ^{-/-} MCps expressing p85 α Δ SH3 or p85 α Δ BH. These results suggest that cooperation between the SH3 and BH amino terminal domains of full-length p85 α is required for full activation of signaling downstream of the c-Kit receptor.

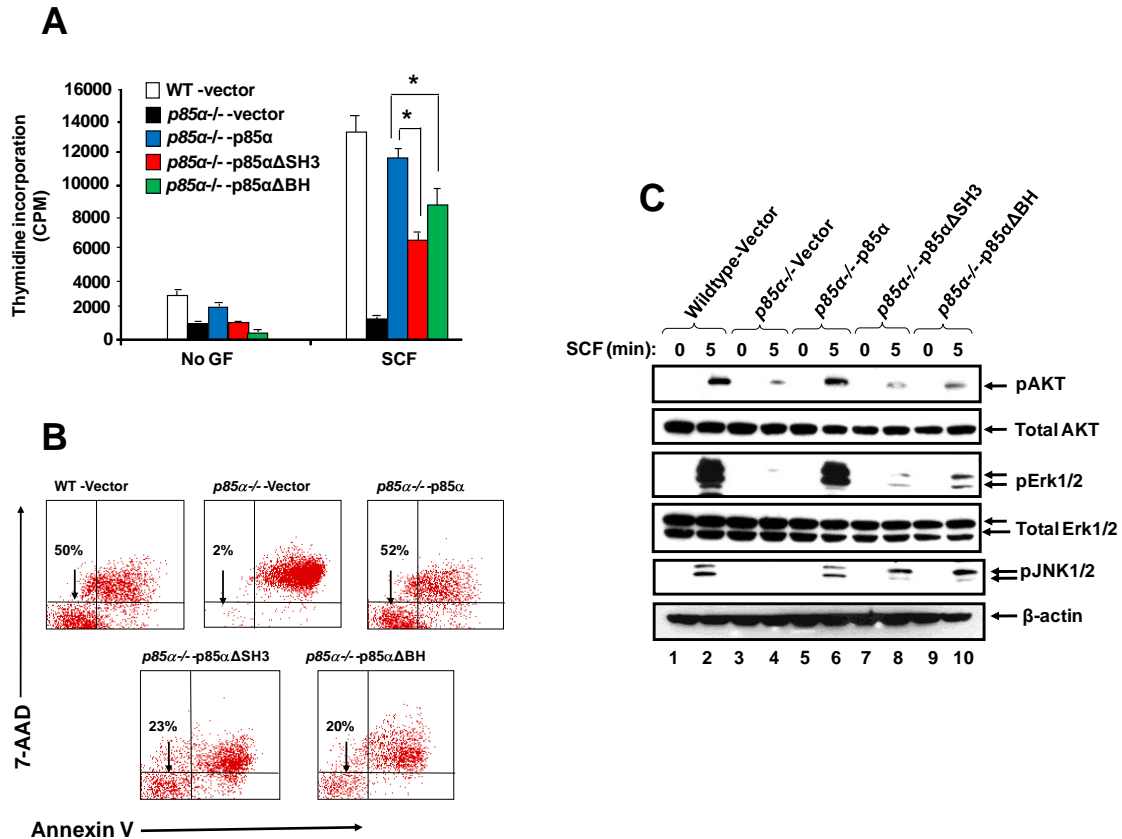


Figure 21. The SH3 and BH domains of *p85α* are important for SCF-induced

growth and survival of mast cells LDBMCs from WT and *p85α*^{-/-} mice were

transduced with vector, full-length *p85α* or *p85α* mutants lacking either the SH3 domain (*p85α*ΔSH3) or the BH domain (*p85α*ΔBH). Cells were sorted to homogeneity and grown in the presence of IL-3 (10 ng/ml). Cells were starved for 6 h in serum- and cytokine-free media and cultured in the presence or absence of SCF (50 ng/ml). **(A)** After 48 h, proliferation was evaluated by [³H] thymidine incorporation. Bars represent the mean [³H] thymidine incorporation (CPM ± S.D) from one representative experiment performed in quadruplicate. Similar results were observed in several independent experiments.

p*<0.01. **(B) After 48 h, cells were stained with PE-conjugated-Annexin V and 7-AAD, and analyzed by flow cytometry. A representative dot blot shows the percent survival of

BMMCs (lower-left coordinate) as determined by the lack of staining of either Annexin V and/or 7-AAD. **(C)** Transduced and sorted cells grown in the presence of IL-3 (10 ng/ml) were starved overnight in serum- and cytokine-free media and stimulated with SCF (100 ng/ml) for 5 min. Cells were harvested and equal amounts of protein lysates were subjected to Western blot analysis using an anti-phospho- AKT antibody, anti-phospho- ERK1/2, or anti-phospho-JNK1/2, as indicated. The amount of total AKT and total ERK proteins were also measured by western blotting. β -actin was used as a loading control for the western analysis.

F. p85 α mutant constructs bind to Gab1, Gab2, and Rac2 upon SCF stimulation

Although mutant p85 α constructs (p85 α Δ SH3 and p85 α Δ BH) restored c-Kit expression and bound to activated c-Kit upon SCF stimulation (Figure 22B), the absence of the SH3 or BH domains of p85 α significantly reduced the growth and survival of mast cells, and significantly reduced activation of AKT, ERK and JNK1 in response to SCF stimulation. To understand the molecular basis for these signaling events, we used the 32D myeloid cell line to evaluate full-length and mutant p85 α constructs for their interactions with other proteins of known importance to mast cell functions when stimulated with SCF. Because 32D cells do not have endogenous c-Kit expression, we co-infected 32D cells with c-Kit and the WT and mutant p85 α constructs. Figure 22A shows the expression of various constructs in 32D cells as assessed by Western blots, using an anti-HA antibody and a p85 α -specific antibody. Immunoprecipitation experiments were performed using lysates prepared from the 32D cells expressing HA-tagged versions of p85 α , p85 α Δ SH3 or p85 α Δ BH. The proteins in the immunocomplexes were then analyzed by Western blot, using an antibodies recognizing Gab1, Gab2, Grb2 or Rac2. As seen in Figure 23, both full-length and mutant p85 α constructs bound to these molecules upon SCF stimulation.

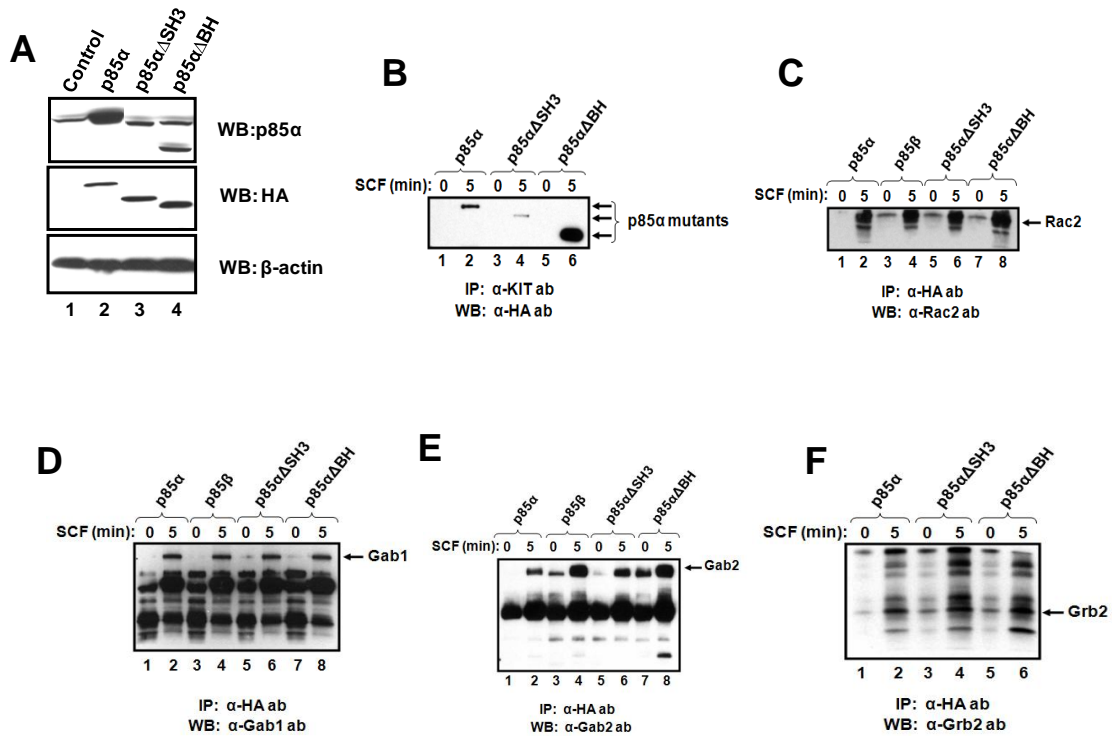


Figure 22. p85α mutant constructs bind to Gab1, Gab2, and Rac2 upon SCF

stimulation (A) Western blot analyses of lysates prepared from the 32D cells show expression of p85α expression (top panel), and the various HA-tagged constructs using anti-HA antibody (middle panel). The levels of protein in each lane were normalized by the amount of β-actin (bottom panel). 32D cells co-infected with c-Kit and p85 constructs were starved and stimulated with SCF (100 ng/ml) for 5 min. **(B)** Equal amounts of cell lysates were subjected to immunoprecipitation with anti-c-Kit antibody, followed by Western blot analysis with an anti-HA antibody. Equal amounts of cell lysates were subjected to immunoprecipitation with anti-HA antibody, followed by Western blot analysis with **(C)** anti-Gab1 antibody, **(D)** an anti-Gab2 antibody, **(E)** an anti-Grb2 antibody or **(F)** anti-Rac2 antibody.

VII. Deficiency of p85 α alters the gene expression profile of cultured mast cells

Our results suggest that the p85 α regulatory subunit of PI-3K plays critical role in maturation, proliferation, survival and cell cycle of mast cells. To explain the underlying molecular mechanism for the action of p85 α , we performed microarray analyses to compare the gene expression profile of bone-marrow–derived WT- and p85 α -deficient mast cells upon stimulation with SCF. Agilent whole mouse genome oligo microarrays (4 x 44K) were used to profile gene expression using RNA purified from the WT and p85 α ^{-/-} MCps.

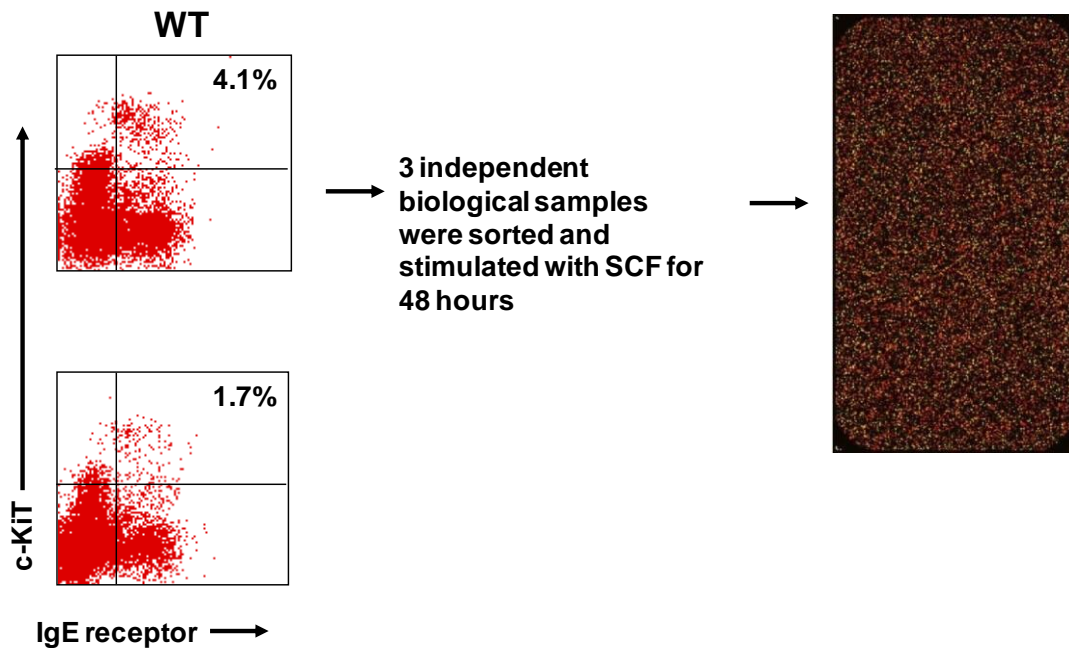


Figure 23. Methodology followed for microarray analysis. Low-density bone marrow cells collected from three independent biological samples of WT and p85 α ^{-/-} mice were cultured in the presence of IL-3 (10 ng/ml) for 1 week. FACS was used to sort the mast cells that were double-positive for c-Kit and IgE expression. Double-positive cells were

then stimulated with 100 ng/ml SCF for 48 h. These samples were then sent for microarray analysis for gene expression profiling.

The quality of the microarray results was assessed by performing a Principal Component Analysis (PCA) using Partek software. PCA was applied to the expression data with WT and *p85α^{-/-}* genotypes as variables, and the gene expression measurements as observations. Based on gene expression values, Eigen values and Eigen vectors were calculated, and spatial distribution of each treatment group (genotype) was plotted on a 3D scatter plot. Statistically, we observed good spatial separation between the samples, which indicates a low correlation between the two groups of samples (WT and *p85α^{-/-}*). Low variance within each group was indicated by close clustering of samples within a group. The results suggested good-quality microarray output that could be analyzed for alteration in gene expression. The data were used for further analysis to investigate underlying molecular mechanism to explain the defective phenotype in *p85α^{-/-}* MCps in response to SCF stimulation.

To determine significantly altered genes in *p85α*-deficient mast cells as compared to WT, we calculated the *p* value for gene expression pattern based on Student's t-test using Partek software with the following criteria: change in expression of at least 1.5-fold and *p*<0.05. This analysis identified 151 unique genes exhibiting altered expression between WT and *p85α^{-/-}* cells (Table 1).

To address the potential role of these changes in gene expression in mast cell biology, we collated these genes into functional categories using the DAVID bioinformatics tool, and the Ingenuity Pathway Analysis (IPA) software. The DAVID tool relates altered

genes due to lack of p85 α to transcription, cell cycle, cell adhesion, cell differentiation, cell survival and signal transduction (Tables 2 and 3).

Most interesting genes from microarray data are - *Rrm1* (Ribonucleotide Reductase M1), which is found to be upregulated in *p85 α -/-* MCps in response to SCF stimulation.

Upregulation of *Rrm1* might be partly contributing to significantly reduced PI-3K activity in *p85 α -/-* MCps upon SCF stimulation, as this gene is report to induce the expression of PI-3K negative regulator PTEN (Gautam, Li et al. 2003). *Taok3* (Tao kinase 3) and *Fbln5* (Fibulin5) which has been reported to activate ERK1/ERK2, and is down regulated in *p85 α -/-* MCps in response to SCF stimulation (Zhang, Chen et al. 2000). These genes might be partly regulating the ERK activation downstream of p85 α ; the downregulation of which results in significantly reduced growth in reponse to SCF stimulation in *p85 α -/-* MCps. *Pim3* (Pim3 kinase) has been reported to positively regulate cell proliferation and survival via activation of BIM (Macdonald, Campbell et al. 2006). Down regulation of *Pim3* might contribute to significantly increased apoptosis of p85 α -deficient cells. These information could be evaluated in future study in context of explaining the growth and survival defects in *p85 α -/-* MCps upon SCF stimulation.

Table 1. List of of genes altered in $p85\alpha^{-/-}$ in response to SCF stimulation

Gene symbol	Gene description	Fold Change	p-value
4930447A16Rik	RIKEN cDNA 4930447A16 gene	-7.69	0.02
Cdh8	Cadherin 8	-7.09	0.05
Atpbd4	ATP binding domain 4	-6.37	0.008
1810047C23Rik	RIKEN cDNA 1810047C23 gene	-6.12	0.03
Qsox1	Quiescin Q6 sulfhydryl oxidase 1	-5.58	0.03
A030006P16Rik	Keratin associated protein 17-1	-5.21	0.024
NAP069078-1	NAP069078-1	-4.64	0.001
1700017D01Rik	1700017D01Rik	-4.63	0.01
AK047500	AK047500	-4.5	0.007
Laptm4b	Lysosomal-associated protein transmembrane 4B	-4.13	0.005
9330155M09Rik	RIKEN cDNA 9330155M09 gene	-3.85	0.02
Txndc5	Thioredoxin domain containing 5	-3.72	0.001
Pim3	Proviral integration site 3	-3.64	0.000904
Tcf20	Transcription factor 20	-3.5	0.01
Taok3	TAO kinase 3	-3.41	0.02
TC1531727	TC1531727	-3.38	0.02
4922502D21Rik	RIKEN cDNA 4922502D21 gene	-3.33	0.02
Ubl4b	Ubiquitin-like 4B	-3.32	0.03
Slc1a1	Solute carrier family 1	-3.28	0.05
D330028D13Rik	RIKEN cDNA D330028D13 gene	-3.15	0.01
1700069L16Rik	RIKEN cDNA 1700069L16 gene	-3.09	0.02
Uimc1	Ubiquitin interaction motif containing 1	-3.06	0.02
Cbx5	Chromobox homolog 5	-2.95	0.04
Bpgm	2,3-bisphosphoglycerate mutase	-2.95	0.03
1200009I06Rik	RIKEN cDNA 1200009I06 gene	-2.93	0.02
Dus1l	Dihydrouridine synthase 1-like	-2.86	0.02
A_52_P868798	A_52_P868798	-2.68	0.03
Pofut1	Protein O-fucosyltransferase 1	-2.67	0.04
D830039M14Rik	RIKEN cDNA D830039M14 gene	-2.58	0.04
BC049715	Hypothetical protein LOC100044248	-2.54	0.02
AB182283	AB182283	-2.48	0.05
2310040A13Rik	WD repeat domain 20A	-2.44	0.02
Nbn	Nibrin	-2.41	0.02
Olf414	Olfactory receptor 414	-2.31	0.03
NAP123291-1	NAP123291-1	-2.28	0.04
ENSMUST00000101328	ENSMUST00000101328	-2.28	0.03
Sema3d	Sema domain, immunoglobulin domain	-2.26	0.04

Gene symbol	Gene description	FoldChange	p-value
1700023L04Rik	RIKEN cDNA 1700023L04 gene	-2.21	0.04
Slc25a10	Solute carrier family 25, member 10	-2.21	0.03
Arhgef3	Rho guanine nucleotide exchange factor (GEF) 3	-2.19	0.05
1700021K19Rik	RIKEN cDNA 1700021K19 gene	-2.18	0.03
Dyrk1a	Dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 1a	-2.13	0.04
Nsd1	Nuclear receptor-binding SET-domain protein 1	-2.12	0.03
Abca12	ATP-binding cassette, sub-family A (ABC1)	-2.11	0.02
Defb41	Defensin beta 41	-2.11	0.02
Deaf1	Deformed epidermal autoregulatory factor 1	-2.1	0.02
D930016D06Rik	RIKEN cDNA D930016D06 gene	-2.1	0.02
Mbnl1	Muscleblind-like 1	-2.04	0.009
AK087625	AK087625	-2.04	0.04
Stard3	START domain containing 3	-2.03	0.02
Zfp667	Zinc finger protein 667	-2.02	0.04
Gls	Glutaminase	-1.97	0.04
Caln1	Calneuron 1	-1.92	0.04
Fbxo3	F-box protein 3	-1.91	0.04
Pcgf2	Polycomb group ring finger 2	-1.9	0.02
F730031O20Rik	Cas scaffolding protein family member 4	-1.88	0.02
ENSMUST00000061772	ENSMUST00000061772	-1.86	0.009
AK085452	Predicted gene 9973	-1.84	0.017
Nudt16l1	Nudix type motif 16-like 1	-1.77	0.02
Psme4	Proteasome activator subunit 4	-1.77	0.04
4933404M19Rik	RIKEN cDNA 4933404M19 gene	-1.77	0.04
Rp2h	Retinitis pigmentosa 2 homolog	-1.75	0.02
Rsad2	Radical S-adenosyl methionine domain containing 2	-1.74	0.02
Arntl2	Aryl hydrocarbon receptor nuclear translocator	-1.73	0.01
LOC620807	LOC620807	-1.72	0.04
Mov10l1	Moloney leukemia virus 10-like 1	-1.7	0.04
Pex11c	Peroxisomal biogenesis factor 11 gamma	-1.7	0.03
Zdhhc5	Zinc finger, DHHC domain containing 5	-1.68	0.02
Olf309	Olfactory receptor 309	-1.66	0.01
Zfp592	Zinc finger protein 592	-1.66	0.02
Btn1a1	Butyrophilin, subfamily 1, member A1	-1.65	0.005
Fbln5	Fibulin 5	-1.64	0.02
2310021P13Rik	RIKEN cDNA 2310021P13 gene	-1.64	0.02
Ubie	Methyltransferase like 7A2	-1.63	0.04
P4ha3	Procollagen-proline, 2-oxoglutarate 4-dioxygenase alpha polypeptide III	-1.59	0.03

Gene symbol	Gene description	FoldChange	p-value
Mobk11a	MOB1, Mps One Binder kinase activator-like 1A	1.77	0.01
Smgc	Submandibular gland protein C	1.79	0.04
AA536717	AA536717	1.79	0.04
AK033099	AK033099	1.79	0.01
Phip	Pleckstrin homology domain interacting protein	1.82	0.01
Ubr2	Ubiquitin protein ligase E3 component n-recognin 2	1.85	0.05
Trps1	Trichorhinophalangeal syndrome I	1.87	0.02
Phox2a	Paired-like homeobox 2a	1.88	0.03
Sptlc1	Serine palmitoyltransferase, long chain base subunit 1	1.92	0.01
AK033617	AK033617	1.92	0.007
Klk6	Kallikrein related-peptidase 6	1.93	0.02
Prdm4	PR domain containing 4	1.95	0.01
Lyrm5	LYR motif containing 5	1.95	0.04
AK019527	AK019527	1.97	0.01
Bub3	Budding uninhibited by benzimidazoles 3 homolog	2.1	0.01
Sufu	Suppressor of fused homolog	2.1	0.01
Fert2	Fer (fms/fps related) protein kinase, testis specific 2	2.25	0.006
2610020C11Rik	Zinc finger protein 157	2.26	0.03
AK082639	AK082639	2.3	0.01
Agps	Alkylglycerone phosphate synthase	2.3	0.04
Ift81	Intraflagellar transport 81 homolog	2.42	0.03
Ucp2	Uncoupling protein 2	2.43	0.02
AK047379	AK047379	2.45	0.003
A630007B06Rik	RIKEN cDNA A630007B06 gene	2.45	0.03
D930043O14Rik	RIKEN cDNA D930043O14 gene	2.51	0.04
Cetn1	Centrin 1	2.64	0.05
Hisppd2a	Histidine acid phosphatase domain containing 2A	2.66	0.02
Asb5	Ankyrin repeat and SOC box-containing 5	2.8	0.01
Kcnj14	Potassium inwardly-rectifying channel, subfamily	2.88	0.03
4930413O22Rik	RIKEN cDNA 4930413O22 gene	3.34	0.006
AK083897	AK083897	3.48	0.01
Acad11	Acyl-Coenzyme A dehydrogenase family	3.9	0.02
Lin9	Lin-9 homolog	4.17	0.03
Zfp26	Zinc finger protein 26	4.3	0.006
Rnf150	Ring finger protein 150	4.4	0.01
NAP028759-1	NAP028759-1	4.43	0.005
2610016C23Rik	Family with sequence similarity 54, member A	4.58	0.01
Ttc29	Tetratricopeptide repeat domain 29	4.65	0.002
1110032A13Rik	RIKEN cDNA 1110032A13 gene	6.83	0.04

Table 2. Functional categories of genes upregulated in $p85\alpha^{-/-}$ in response to SCF stimulation

Functional category	Gene symbol	Gene name	Fold Change
Ubl conjugation pathway	Senp6	Sumo/Sentrin Specific Peptidase 6	1.62
	Asb5	Ankyrin Repeat And Socs Box-Containing Protein	2.8
	Ubr2	Riken Cdna E130209G04 Gene	1.84
Proteolysis	Senp6	Sumo/Sentrin Specific Peptidase 6	1.62
	Ubr2	Riken Cdna E130209G04 Gene	1.84
	Klk6	Kalikrein 6	1.92
	Rnf150	Ring Finger Protein 150	4.39
Cell surface receptor	Il23r	Interleukin 23 Receptor	1.74
	Olf1107	Olfactory Receptor 1107	1.73
	Sufu	Suppressor of Fused Homolog	2.11
	Olf1179	Olfactory Receptor 1179	1.58
	Gpr21	G Protein-Coupled Receptor 21	1.57
Cell cycle	Bub3	Budding Uninhibited By Benzimidazoles 3 Homolog	2.09
	Ubr2	Riken Cdna E130209G04 Gene	1.84
	Lin9	Lin-9 Homolog (C. Elegans)	4.16
	Cetn1	Centrin 1	2.63
	Atp6v1b2	Atpase, H ⁺ Transporting, Lysosomal V1 Subunit B2	1.67
	Rrm1	Ribonucleotide Reductase M1	1.67
	Agps	Alkylglycerone Phosphate Synthase	2.3
	Cetn1	Centrin 1	2.63

Table 3. Functional categories of genes downregulated in *p85α*^{-/-} in response to SCF stimulation

Functional category	Gene symbol	Gene name	Fold Change
Transcription regulation	Nsd1	Nuclear receptor-binding set-domain protein 1	-2.11
	Amtl2	Aryl hydrocarbon receptor nuclear translocator-like	-1.72
	Pcgf2	Polycomb group ring finger 2	-1.9
	Tcf20	Transcription factor 20	-3.5
	Pou4f3	Pou domain, class 4, transcription factor 3	-1.55
	Uimc1	Retinoid x receptor interacting protein 110	-3.06
Protein kinase activity	Pim3	Proviral integration site 3	-3.63
	Taok3	Tao kinase 3	-3.41
	Dyrk1a	Dual-specificity tyrosine phosphorylation regulated kinase 1a	-2.13
Cell differentiation	Sema3d	Riken cDNA 4631426b19 gene	-2.26
	Psme4	Proteasome activator subunit 4	-1.77
	Mbnl1	Muscleblind-like 1 (drosophila)	-2.03
	Pou4f3	Pou domain, class 4, transcription factor 3	-1.55
Cell adhesion	Cdh8	Cadherin 8	-7.09
	Fbln5	Fibulin 5	-1.64
	Col2a1	Procollagen, type ii, alpha 1	-1.56
Signal transduction	Pofut1	Protein o-fucosyl transferase 1	-2.66
	Amtl2	Aryl hydrocarbon receptor nuclear translocator-like 2	-1.72
	Taok3	Tao kinase 3	-3.41
	Arhgef3	Rho guanine nucleotide exchange factor 3	-2.18
	Rabl2a	Rab, member of ras oncogene family-like 2a	-1.58
Post-translational protein modification	Nsd1	Nuclear receptor binding set domain protein 1	-2.11
	Pim3	Proviral integration site 3	-3.63
	Taok3	Tao kinase 3	-3.41
	Pcgf2	Polycomb group ring finger 2	-1.9
	Fbxo3	F-box only protein 3	-1.9
	Dyrk1a	Dual-specificity tyrosine phosphorylation regulated kinase 1a	-2.13

We performed Ingenuity Pathway Analysis (IPA) to investigate the associated biological pathways which are altered in the absence of p85 α in mast cells. IPA is a curated database that provides information on biological functional networks, based on published scientific literature. Figure 24 shows relevant networks altered in p85 α -deficient cells, which are applicable to this study.

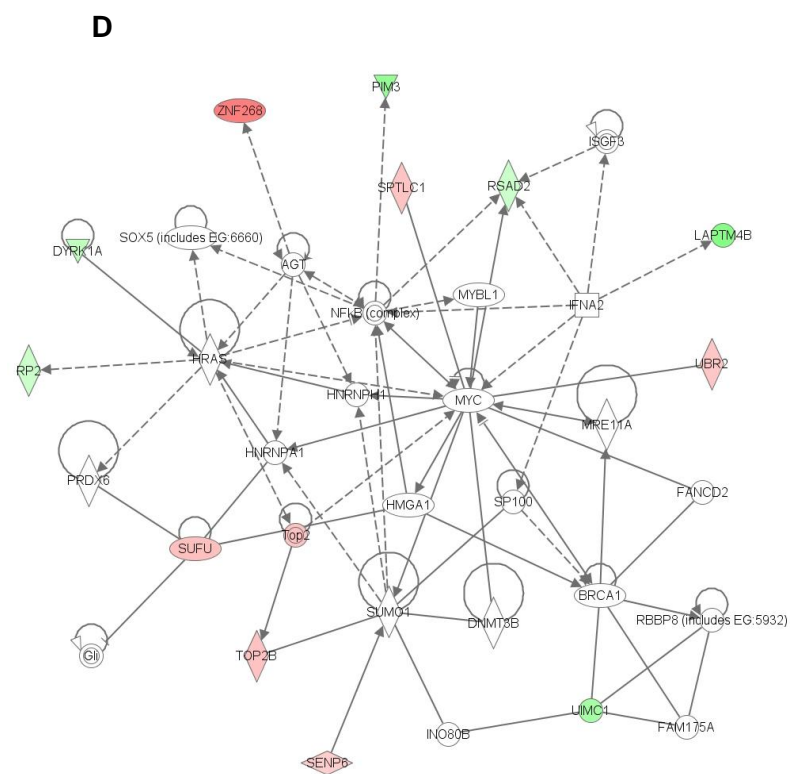
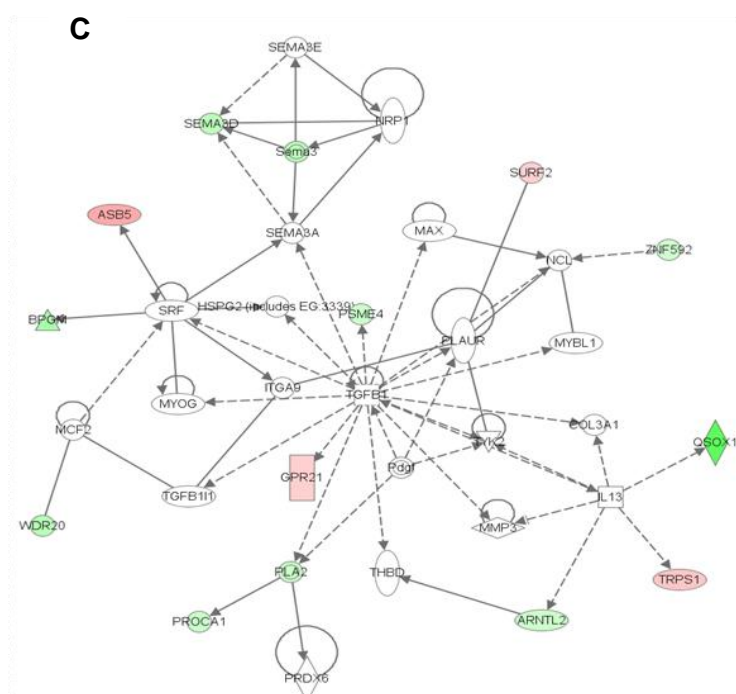


Figure 24. IPA results on functional network of significantly altered genes in $p85\alpha^{-/-}$ cells in response to SCF stimulation. Altered genes in $p85\alpha^{-/-}$ cells after SCF stimulation were analyzed by Ingenuity Pathway Analysis (IPA) software to analyze their closely associated gene network. Interestingly, significantly altered gene in $p85\alpha^{-/-}$ were associated with **(A)** cell survival, **(B)** cell growth, **(C)** cell migration and **(D)** the cell cycle.

Identification of these four principal networks is relevant to this study, as our functional data suggest that mast cells lacking the $p85\alpha$ regulatory subunit display defective cell cycles, altered survival and differentiation. The IPA network provides further information on additional genes including *Pdgfb*, *AKT*, *Hnf4A*, *Stat3*, *Tgfb*, *Myc*, *Brca1*, *Crebbp*. These genes identified by IPA tool, which are related to the altered genes in $p85\alpha^{-/-}$ per the microarray data, could also play roles in mast cell biological functions in response to SCF stimulation.

VIII. PI-3K regulatory subunits $p85\alpha$ and $p85\beta$ differentially regulate mast cell development *in vivo*

To confirm our *in vitro* data, *in vivo*, we transplanted $W^{sh/sh}$ recipient mice, with $p85\alpha^{-/-}$ stem cells transduced with various $p85\alpha$ constructs. $p85\alpha$ -deficient bone marrow cells treated with 5-FU (5-fluorouracil), was transduced with bicistronic MIEG3 expression plasmids encoding $p85\alpha$, $p85\beta$, $p85\alpha\Delta SH3$ or $p85\alpha\Delta BH$, cells were then sorted for homogeneity based on EGFP expression and transplanted into $W^{sh/sh}$ mice. We hypothesized that because $W^{sh/sh}$ mice lack mast cells, any mast cell population generated in the transplanted mice should be due to the activity of the donor cells'. After transplantation, we monitored the homing efficiency of the donor cells in the transplanted mice on a monthly basis by evaluating EGFP expression in their peripheral blood. After 4 months, we sacrificed the $W^{sh/sh}$ recipient mice and harvested tissues from each mouse.

These tissues, which included the ear, skin, lungs, spleen, stomach, duodenum, ileum, jejunum and colon, were subsequently analyzed for mast cell reconstitution in various tissues.

Representative spleen and stomach (Figure 25) sections of $W^{sh/sh}$ mice transplanted with p85 α -deficient cells infected the WT or mutant p85 constructs are shown, with mast cells indicated by arrows. Consistent with our *in vitro* studies, full-length p85 α transduced into $p85\alpha^{-/-}$ BM cells fully reconstituted the mast cell population in $W^{sh/sh}$ transplanted mice, where as p85 α mutants p85 $\alpha\Delta$ SH3 and p85 $\alpha\Delta$ BH partially reconstituted mast cells. In contrast, p85 β -expressing $p85\alpha^{-/-}$ BM cells showed only minimal reconstitution of mast cells (Figure 26). These results confirm the differential regulation of mast cell development by PI-3K regulatory subunits p85 α and p85 β *in vivo*, and the importance of the SH3 and BH domains of p85 α in normal mast cell development.

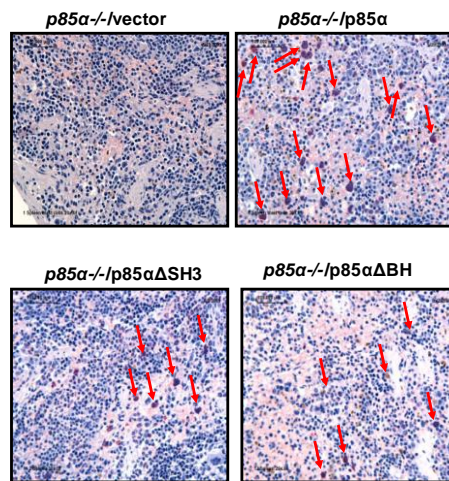
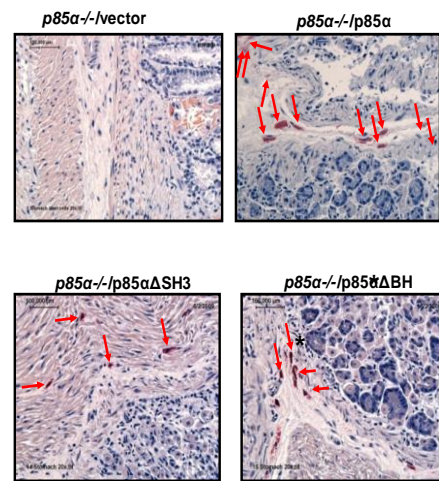
A**B**

Figure 25. The amino terminal SH3 and BH domains of p85 α are critical for mast cell development *in vivo*. p85 α ^{-/-} mice were injected intraperitoneally with 5-FU (150 mg/kg body weight) and LDBMCs were harvested 72 h after injection. LDBMCs were transduced with full-length p85 α or p85 α mutants (p85 α ΔSH3 or p85 α ΔBH) and sorted to homogeneity according to EGFP expression. Sorted cells (1×10^6 cells) were mixed with recipient $W^{sh/sh}$ splenocytes (0.1×10^6 cells) and injected into mast-cell-deficient $W^{sh/sh}$ mice. Four months after transplantation, the mice were sacrificed; different tissues were harvested and analyzed for mast cells by toluidine staining. Shown are representative sections of spleen **(A)** and stomach **(B)**.

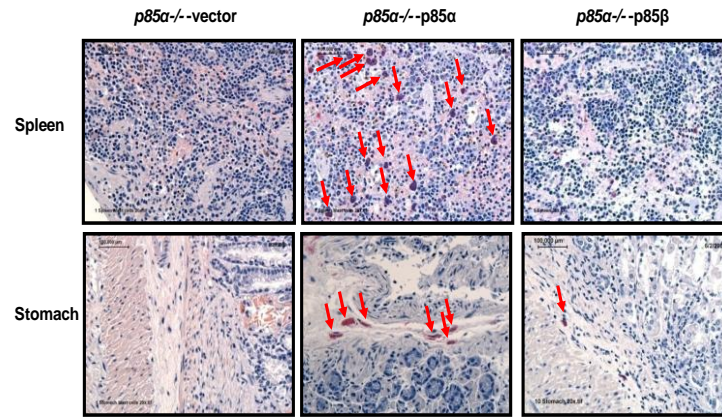


Figure. 26. PI-3K regulatory subunits p85 α and p85 β differentially regulates mast cell development *in vivo*. Histological analysis of stomach and spleen showing the reconstitution of mast cells in W^{sh} mice. $p85\alpha^{-/-}$ mice were injected intraperitoneally with 5-flurouracil (150 mg/kg body weight) and BM cells were harvested after 72 hours of injection. These cells were transduced with p85 α or p85 β and sorted to homogeneity on the basis of EGFP expression. Sorted cells (1×10^6) were mixed with recipient W^{sh} splenocytes (0.1×10^6 cells) and injected into mast cell deficient W^{sh} mice. After 4 months of transplantation, mice were sacrificed; different tissues were harvested and analyzed for mast cells by leader staining. Shown are representative sections of spleen and stomach from the indicated genotypes. Arrows indicate mast cells in various tissues.

DISCUSSION

Previous studies have demonstrated the crucial role of the c-Kit receptor and its ligand SCF in mast cell development (Kitamura, Go et al. 1978; Galli and Kitamura 1987; Chabot, Stephenson et al. 1988; Copeland, Gilbert et al. 1990; Huang, Nocka et al. 1990; Martin, Suggs et al. 1990; Matsui, Zsebo et al. 1990; Nocka, Buck et al. 1990; Williams, Eisenman et al. 1990; Zsebo, Williams et al. 1990; Zsebo, Wypych et al. 1990; Tsai, Shih et al. 1991). PI-3K, which is activated by binding to the 719Y residue of c-Kit, is a critical mediator in transmitting signals downstream of c-Kit. (Shivakrupa, Bernstein et al. 2003). PI-3K is also thought to be a critical mediator of abnormal c-Kit signaling, due to c-Kit point mutation (c-Kit D816V in human and c-Kit D814V in mouse), which leads to clinical symptoms, such as mastocytosis and mast cell leukemia (Nagata, Worobec et al. 1995; Longley, Tyrrell et al. 1996; Longley, Metcalfe et al. 1999; Longley, Reguera et al. 2001). Pharmacological inhibitors of PI-3K activity and dominant negative inhibitory PI-3K proteins provide evidence for the crucial role of PI-3K in mediating ligand-independent abnormal c-Kit signaling (Munugalavadla, Sims et al. 2007).

Class IA PI-3K is a complex of regulatory (p85) and catalytic subunit (p110), the latter of which is activated upon binding to c-Kit via its regulatory subunit. Based on recent studies demonstrating enhanced insulin sensitivity in both *Pik3r1*^{-/-} and *Pik3r2*^{-/-} mice, it is generally believed that all regulatory subunits of class IA PI3K behave in a functionally redundant manner with their primary function being to stabilize and activate the different p110 catalytic subunits (Terauchi, Tsuji et al. 1999; Fruman, Mauvais-Jarvis et al. 2000; Ueki, Yballe et al. 2002; Chen, Mauvais-Jarvis et al. 2004).

In this study, we evaluated the role of p85 α regulatory subunit in mast cell growth, maturation and survival. We showed that genetic disruption of p85 α results in defective

IL-3 mediated mast cell differentiation and c-kit mediated growth and survival, irrespective of the presence of other PI-3K regulatory subunits; p85 β , p55 α and p50 α . p85 α being the most abundantly expressed regulatory subunit in mast cells, its genetic disruption might significantly reduce the total amount and balance of PI-3K regulatory subunits overall, which might be the reason for defective phenotype in *p85 α ^{-/-}*. We evaluated this possibility by performing reconstitution studies, and observed that the reconstitution of *p85 α ^{-/-}* MCps with p85 α but not p85 β completely corrects both IL-3-mediated differentiation as well as c-Kit induced proliferation. This suggests that the phenotypic defects observed in *p85 α ^{-/-}* MCps are a result of specific loss of p85 α (full-length) and not due to quantitative reduction in the overall expression of regulatory subunits.

Given the fact that p85 β cannot substitute for the loss of p85 α in MCps, we then evaluated whether these regulatory subunits have differential roles in mast cell biological functions in response to SCF and IL-3 stimulation. Our results in primary hematopoietic cells using both genetic and biochemical approaches clearly demonstrate unique role(s) for p85 regulatory subunits in mast cell growth, maturation and survival. We show that genetic disruption of p85 α (full-length) results in defective IL-3 mediated mast cell differentiation and c-Kit mediated growth and survival; whereas genetic disruption of p85 β results in enhanced IL-3 mediated c-Kit expression; and enhanced c-Kit mediated growth and survival. Significantly impaired response of *p85 α ^{-/-}* MCps to SCF stimulation might be due to reduced surface c-Kit expression in these cells, or might be due to an intrinsic defect due to loss of p85 α regulatory subunit. We evaluated the possibilities performing thymidine incorporation assay on c-Kit sorted WT and *p85 α ^{-/-}* MCps and confirmed that the defective mast cell growth in response to SCF stimulation is intact due to the loss of p85 α regulatory subunit.

SCF stimulation of c-Kit signaling initiate activation of several ubiquitin ligases that terminate c-Kit signals in normal cells (Masson, Heiss et al. 2006). Among those ligases is c-Cbl; its direct binding contributes to c-Kit receptor ubiquitination, internalization and degradation (Masson, Heiss et al. 2006). Ubiquitination and subsequent degradation of c-Kit has been implicated as the key mechanism in regulating the duration and intensity of downstream signaling events (Miyazawa, Toyama et al. 1994). Our results show impaired c-Kit receptor internalization in *p85 β* ^{-/-} MCps compared to WT controls. Consistently, we show that overexpression of p85 β enhances the rate of c-Kit internalization and degradation compared to cells expressing p85 α subunit. These results suggest that the enhanced proliferation and survival of *p85 β* ^{-/-} MCps in response to SCF is likely due to reduced c-Kit receptor internalization leading to prolonged activation of downstream signals including ERK and JNK MAP kinase. E3 ubiquitin ligases including Cbl proteins are involved in binding and degradation of activated c-Kit leading to down-regulation of c-Kit receptor signaling (Zeng, Xu et al. 2005). We observed preferential binding of p85 β to c-Cbl compared to p85 α in c-Kit stimulated cells, which was associated with enhanced c-Kit receptor ubiquitination and degradation. Our data is in agreement with previous study done in T-cell, where p85 β was reported to preferentially bind to c-Cbl via its N-SH2 and SH3 domain in modulating T-cell functions (Alcazar, Cortes et al. 2009). This function of p85 β explains the reduced c-Kit internalization, and enhanced c-Kit expression as well as enhanced signaling events downstream of c-Kit in *p85 β* ^{-/-} MCps. Thus, p85 β through interaction with c-Cbl regulates the internalization and degradation of c-Kit, deficiency of which is likely to contribute to enhanced c-Kit induced growth, survival and activation of downstream signals.

p85 α and p85 β regulatory subunits of class IA PI3-K share near identity in the known functional domains in the carboxy terminus, including the amino-SH2 and the carboxy-SH2 domains, but wide disparity in amino acid composition exists in the amino terminal domains of SH3 and BH. Interestingly p85 α splice variants - p50 α and p55 α share the domains in the carboxy terminus, but lack amino terminal domains of SH3 and BH. Therefore the basis for functional uniqueness of p85 α is likely to be derived from the amino terminus SH3 and BH domains of this regulatory subunit. The notion of specificity was further exemplified in studies demonstrating only a partial rescue in c-Kit induced growth and survival in *p85 α -/-* MCps reconstituted with p50 α , p85 $\alpha\Delta$ SH3 or p85 $\alpha\Delta$ BH. While our studies show high degree of specificity by various subunits in regulating c-Kit induced functions; the role in IL-3 induced maturation of MCps is somewhat redundant. Overexpression of p50 α or p85 $\alpha\Delta$ SH3 or p85 $\alpha\Delta$ BH in *p85 α -/-* MCps completely restores maturation as well as the surface c-Kit expression to WT levels. Restoration of c-Kit expression in these cells might be either due to restored expression of a transcription factor—Mitf, which enhances c-Kit protein expression in these cells; or due to inability of the p85 $\alpha\Delta$ SH3 or p85 $\alpha\Delta$ BH constructs to bind to ubiquitinating ligase c-Cbl, which hinders c-Kit internalization and degradation.

Mitf is a key regulator of mast cell maturation. Mitf regulates a number of proteases, cell surface receptors, signaling molecules and transporters critical for mast cell function (Shahlaee, Brandal et al. 2007). Although Mitf upregulation is essential during early stages of mast cell maturation, the molecular mechanism regulating Mitf expression in mast cell maturation is poorly understood. Here we observe a possible role of p85 α regulatory subunit of PI-3K in IL-3 mediated Mitf regulation in MCps, which in turn partly affects c-Kit expression and maturation of mast cells. Mitf expression was abolished in absence of p85 α regulatory subunit in MCps. Restoration of full length p85 α in *p85 α -/-*

MCps restores the expression of Mitf to WT levels. The result suggests that Mitf expression in mast cells in response to IL-3 stimulation is tightly regulated by p85 α regulatory subunit of PI-3K.

Earlier studies report the regulational role of Mitf on c-Kit expression; and a significant down regulation of c-Kit expression is observed in mast cells from Mitf mutant mice (Ebi, Kanakura et al. 1992; Isozaki, Tsujimura et al. 1994). Consistently in this study, the down regulation of Mitf expression coincided with significant reduction in c-Kit expression in *p85 α -/-* MCps. Further the restoration of full length p85 α in *p85 α -/-* MCps which restores the expression of Mitf, also restored c-Kit expression to WT levels; which suggests involvement of p85 α regulatory subunit in the process. Amino terminal SH3 and BH domains of p85 α might not be involved in the process, as p50 α or p85 α mutants which lack SH3 or BH domains restores the expression of Mitf and c-Kit receptor in *p85 α -/-* MCps to WT levels.

PI-3K regulatory subunit p85 α might regulate Mitf expression at different levels, from transcriptional to post-translational modifications. Mitf expression is regulated by multiple signaling pathways which involve signaling molecules such as AKT and ERK (Wu, Hemesath et al. 2000). Mitf is also regulated by a variety of transcription factors like Pax3, CREB, Sox10, Lef1 and BRN-2 (Goding 2000). Our data suggests that AKT activation is significantly reduced in response to SCF stimulation in MCps lacking p85 α regulatory subunit, the activation which was restored upon expression of full length p85 α . Consistently, Mitf expression which was significantly impaired in *p85 α -/-* MCps, was completely restored with full length p85 α expression upon IL-3 stimulation. Involvement of PI-3K and AKT in Mitf regulation could be further evaluated by expressing p110CAAX and activated form of AKT in *p85 α -/-* MCps.

Inter players mediating the AKT and Mitf activation is yet to be evaluated. PI-3K regulated activation of AKT is reported to induce phosphorylation and activation of CREB, which in turn regulates transcription of Mitf. PI-3K might also be involved in AKT mediated inhibition of GSK-3 β expression, which is involved in phosphorylation and activation of Mitf (Takeda, Takemoto et al. 2000). Thus, p85 α regulatory subunit of PI-3K might be involved in transcriptional regulation of Mitf via AKT and CREB and/or GSK-3 β .

SCF signaling pathway in melanocytes is reported to target Mitf simultaneously for activation and proteolytic degradation, which is mediated via ERK activation (Wu, Hemesath et al. 2000). Activation of ERK via c-Kit signaling is reported to increase Mitf transactivation potential (Steingrimsen, Copeland et al. 2004). Phosphorylation and activation of Mitf also leads to its ubiquitination and degradation via ubiquitination ligase Ubc9. p85 α regulatory subunit is critical for ERK activation in mast cells as indicated by significant reduction in ERK activity in MCps lacking p85 α regulatory subunit upon SCF stimulation. ERK activity and Mitf expression was restored in *p85 α -/-* MCps upon restoration of full length p85 α expression. Future experiments using ERK pathway specific inhibitor PD98059 would confirm the involvement of ERK in p85 α regulated Mitf expression in MCps. Although this study evaluates AKT and ERK signaling downstream of c-Kit upon SCF stimulation, Mitf and c-Kit expression was evaluated in *in vitro* MCps cultured in presence of IL-3. Further evaluation is needed on specific effects of IL-3 and SCF signaling in both Mitf expression and AKT and ERK activation.

Along with Mitf, other transcription factors GATA-1, GATA-2, PU.1 play essential roles in mast cell maturation. Different stages of mast cell development are tightly regulated by a molecular balance between different transcription factors. Although GATA-2 is critical for maturation of embryonic stem cells into mast cells, results from our lab demonstrate that

over-expression of GATA-2 represses the Mitf expression and antagonizes the maturation of adult bone marrow derived mast cells; the phenotypic defects similar to p85 α deficiency seen in MCps. Previous studies in adipose tissues report that insulin-dependent phosphorylation of GATA-2 in a PI-3K/AKT dependent manner impairs GATA-2 translocation to nucleus and its DNA binding activity. Taken together, these observations suggest that p85 α regulatory subunit of PI-3K might regulate Mitf expression in mast cell by multiple signaling pathways which might involve multiple signaling molecules and transcription factors.

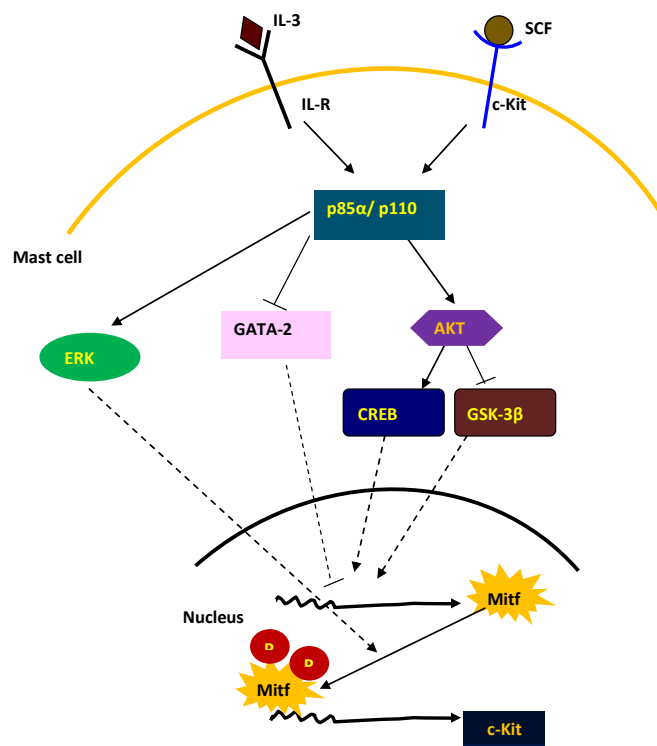


Figure 27 . A model describing the possible mechanisms involved in Mitf regulation by PI3K pathway. Expression of Mitf in MCps might be regulated by multiple signaling pathways involving multiple transcription factors. The above figure represents possible mechanism for Mitf regulation based on information from various previously published literatures.

Although carboxy terminal domains of p85α might be important in mast cell maturation; the growth and survival of mast cells in response to SCF stimulation is critically regulated by amino terminal domains of p85α. We observe that although c-Kit expression was restored in *p85α*^{-/-} MCps expressing p85αΔSH3 or p85αΔBH, signals downstream of c-Kit is still impaired in these cells. These results suggest that the signals emanating from the amino terminus domains of p85α are likely to play a critical role in c-Kit induced mast cell biological functions. To evaluate this, we examined the interaction

of HA-tagged full-length p85 α and mutant p85 α constructs with various proteins known to regulate mast cell growth and survival. We performed immunoprecipitation studies on the 32D mast cell line and examined the interactions of these constructs with proteins including Gab2, Gab1, Rac2 and Grb2.

Gab2 is critical for mast cell proliferation, and its lack of results in severe mast cell deficiency in various tissues (Nishida, Wang et al. 2002; Yu, Luo et al. 2006). Though this adaptor protein is known to bind to the p85 regulatory subunit of PI-3K to bring about this effect (Gu, Pratt et al. 1998; Nishida, Yoshida et al. 1999; Zhao, Yu et al. 1999), here we evaluated how the absence of the SH3 or BH domain of p85 α affects its interaction with Gab2.

Gab1 is a member of Gab/Dos family, which is activated when SCF stimulates c-Kit by SCF. This adaptor protein is linked to ERK kinase activation, which promotes cell survival (Itoh, Yoshida et al. 2000). Even though p85 mutant constructs bound to Gab1 as much as full-length p85 α did upon SCF stimulation, ERK activation was only partially rescued in *p85 α ^{-/-}* MCps expressing the p85 α Δ SH3 or p85 α Δ BH construct.

Grb2 is another scaffolding protein; which regulates cell growth in response to cytokine stimulation-via-activation of Ras signaling (Cutler, Liu et al. 1993). Rac2 is another member of Rho related small GTPase family, that was reported to specifically activate JNK signaling cascade in mast cells (Gu, Byrne et al. 2002). Rac2 deficient mice express severe mast cell deficiency and reduced mast cell survival in response to SCF stimulation (Yang, Kapur et al. 2000). Here we evaluated how the absence of the SH3 or BH domain of p85 α affects its interaction with Grb2 and Rac2.

To determine whether the interactions of the aforementioned proteins were affected by SCF stimulation in the absence of the SH3 or BH domains, we performed functional and immunoprecipitation studies. Using full-length p85 α and mutant p85 α Δ SH3 and p85 α Δ BH, we noted that defective mast cell proliferation and survival in response to SCF stimulation in the absence of an SH3 and/or BH domain is not due to defective interactions with Gab1, Gab2, Grb2, or Rac2 proteins, although they are known to be important effectors of mast cell proliferation and survival. Further, we performed microarray and compared gene expression profiles of WT and p85 α $^{-/-}$ MCps in response to SCF stimulation. Objective was to find potential effectors of PI-3K which might regulate mast cell biological functions upon SCF stimulation. Interesting genes for future consideration in context of explaining the growth and survival defects in p85 α $^{-/-}$ MCps upon SCF stimulation regard are – *Rrm1*, *Taok3*, *Pim3* and *Fbln5*.

Mastocytosis is a myeloid neoplasm characterized by abnormal accumulation and frequent activation of mast cells (MCs) in various organs. The activating point mutation D816V found in the c-Kit kinase domain occurs in 80-95% of systemic mastocytosis (SM) patients. Unfortunately, the prognosis in these patients remains poor and no effective treatment is currently available for the treatment of SM and AML caused due to c-Kit D816V mutations. Through the use of pharmacological inhibitors and genetic studies, PI-3K has been shown to play indispensable role in regulating normal c-Kit signaling, as well as abnormal c-Kit signaling due to D816V mutations. This study was undertaken better understand the role of p85 regulatory subunits and its domains in c-Kit signaling, the information which might be important in designing strategies to regulate abnormal signaling downstream c-Kit D816V.

A redundant functional role of p85 α and p85 β regulatory subunits has been reported by several previous studies (Shepherd, Withers et al. 1998; Ueki, Yballe et al. 2002; Brachmann, Yballe et al. 2005). This study is novel in that it invalidates preexisting assumptions about the functional redundancy or similarity among the various PI-3K regulatory subunits. Performing *in vitro* and *in vivo* studies, we demonstrated that the p85 α and p85 β regulatory subunits of PI-3K play different roles in mast cell development. p85 α is a positive regulator of c-Kit signaling, and the SH3 and BH domains of p85 α were indispensable for the growth of mast cells in response to SCF stimulation. Notably, p85 β appears to be a negative regulator of c-Kit signaling; the absence of p85 β in MCps results in hyperproliferation in response to SCF stimulation, but overexpression of this regulatory subunit in the same cell lines resulted in significantly reduced proliferation and reduced c-Kit expression. Our findings have important implications for the rational design of c-Kit inhibitors, and might be applicable for treatment of malignancies involving abnormal c-Kit 816 mutations, and in tumors involving normal c-Kit signaling.

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Abstracts

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